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**Advancement of a valid model of chronic PTSD and
repetitive mTBI:
Implications for pathophysiology and drug discovery**

A thesis submitted for the degree of Doctor of Philosophy in
the discipline of Neuroscience

Date of submission: November 2018

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Abstract

Post-traumatic stress disorder (PTSD) is increasingly being recognized as a health and economic burden. PTSD can develop after witnessing or experiencing severe or life-threatening trauma and is associated with a number of devastating symptoms that result in decreased quality of life. In addition, PTSD patients have difficulties integrating into society and are at greater risk for other neuropsychiatric disorders, including suicide and substance abuse. The prevalence of PTSD ranges from 8% to 12 % in the general population with a noticeably higher incidences in the US military (20 % -34 %). Current treatment strategies for PTSD are limited and provide only symptomatic relief.

Furthermore, many military-related PTSD cases are complicated with the presence of other comorbidities such as mild traumatic brain injury (mTBI). Comorbid PTSD and mTBI pose particular challenges clinically; the similar symptomatic profiles and unclear presentations in the comorbid condition make it difficult to both diagnose and treat. As both military and civilian populations are increasingly exposed to traumatic stress and mTBI, establishing criteria that facilitate the discrimination between these conditions and developing new effective treatments should be a top priority. Animal models are an appropriate platform for conducting such studies. In Chapter 1, the epidemiology, diagnosis and pathophysiology of PTSD and TBI are reviewed. In addition, a quick summary of preclinical animal models of the two conditions is presented. The thesis then focuses on the establishment and validation of a chronic mouse model of PTSD (Chapter 2) and PTSD with comorbid repetitive mild TBI (Chapters 3). Chapter 4 investigates the key molecular mechanisms implicated in PTSD pathophysiology in a mouse model. Finally, the outcomes of a pilot treatment study are presented in Chapter 5.

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Abbreviations

ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BKY	Benjamini, Krieger and Yekutieli
BOP	Blast overpressure
CAMKII α	Calcium/calmodulin-dependent protein kinase type II alpha chain
CAPS	Clinician-Administered PTSD Scale
CBT	Cognitive behavioral therapy
CCI	Controlled cortical impact
CRH/CRF	Corticotropin-releasing hormone/factor
CRH-R1	CRH receptor type 1
CSF	Cerebrospinal fluid
CT	Computed tomography
CTE	Chronic Traumatic Encephalopathy or CTE
CUS	Chronic unpredictable stress
DAP	3,3'-diaminobenzidine
DEX	Dexamethasone
DG	Dentate gyrus
DHF	7,8 dihydroxyflavone
DNA	Deoxyribonucleic acid
DSM-5	Diagnostic statistical manual version 5
DTI	Diffusion tensor imaging
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EPM	Elevated Plus Maze

FKBP5	FK506 binding protein
fMRI	functional magnetic resonance imaging
FOUR	Full Outline of UnResponsiveness
FPI	Fluid percussion injury
FST	Forced Swim Test
GABA	Gamma-amino butyric acid
GCS	Glasgow Coma Scale
GR	Glucocorticoid Receptor
GRE	Glucocorticoid responsive elements
HPA	Hypothalamic-pituitary adrenal axis
HSP90	Heat shock protein 90
IACUC	Institutional Animal Care and Use Committee
LPS	Lipopolysaccharide
MR	Mineralocorticoid Receptor
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
NPY	Neuropeptide Y
OEF	Operation Enduring Freedom
OIF	Operation Iraqi Freedom
OF	Open Field
OLAW	Office of laboratory animal welfare
PBS	phosphate-buffered saline
PCL-5	The PTSD checklist
PSD 95	Postsynaptic density protein 95
PTSD	Posttraumatic stress disorder
RAWM	Radial Arm Water Maze
r-mTBI	Repetitive mild traumatic brain injury
RM-ANOVA	Repeated measure ANOVA

RUS	Repeated unpredictable stress
SI	Social Isolation
SNP	Single nucleotide polymorphism
SNRI	Serotonin noradrenaline reuptake inhibitors
SPECT	Single-photon emission computed tomography
SPS	Single prolonged stress (SPS)
SSRI	Selective serotonin reuptake inhibitors
TBI	Traumatic brain injury
TMT	Trimethylthiazoline
TrkB	Tropomyosin receptor kinase B
mTBI	Mild traumatic brain injury
UVS	Unpredictable variable stress

Chapter 1. Introduction

1. 1 Incidence of PTSD, TBI and their comorbidity in civilian and combat populations

Posttraumatic stress disorder (PTSD) is typically caused by exposure to a severe life-threatening traumatic event that results in a cluster of debilitating neurobehavioral symptoms (Kennedy, 2007). In the United States, it is estimated that about 13% of women and 6% of men exposed to traumatic events will develop PTSD (Breslau et al. 1999). In the United Kingdom, 3.7% of men and 5.1% of women exposed to traumatic events screened positive for PTSD (Fear et al. 2012).

The incidence of PTSD ranges from 3 to 30 percent in groups exposed to specific traumatic incidents such as war, torture, or rape (Kennedy 2007). Moreover, PTSD prevalence is estimated to increase to over 15% in refugees and conflict-afflicted populations because of exposure to torture and potentially traumatic events (Richter-Levin, Stork, and Schmidt 2018). PTSD is also a common neuropsychiatric disorder among military populations due to the repeated exposure to horrific combat-related events. The prevalence of PTSD in soldiers returning from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) ranges from 4% to 17% for those in the United States (Hines et al. 2014; Hoge et al. 2008) and 3% to 6% for the returning UK veterans (Richardson, Frueh, and Acierno 2010). The probability of developing PTSD is higher for soldiers in combat roles and has been reported to double compared to subjects in noncombat roles (Kok et al. 2012). Furthermore, many soldiers are also exposed to various degrees of traumatic brain injuries (TBI), which can further complicate diagnosis and treatment outcome (Kennedy 2007; Tanev et al. 2014).

A report from the RAND corporation in 2009 indicated that 19 % of the soldiers returning from deployment in Iraq and Afghanistan were diagnosed with TBI and 5% were diagnosed with co-morbid PTSD and TBI (Holdeman 2009). Mild TBI (mTBI) is the most common form of TBI (representing more than 80% of all TBI cases) and often co-exists with other psychiatric conditions such as depression, PTSD and suicidal ideation (Bigler and Tsao 2017; Lindquist, Love, and Elbogen 2017). A number of studies have suggested that risk of developing PTSD increases after mTBI in both civilian (Bryant et al. 2009) and military populations (Kennedy et al. 2010). However, contradictory findings were reported in a study of 781 injured military service members, showing lower rates of PTSD and mood/anxiety disorders in those with mild and moderate-severe TBI (MacGregor et al. 2010).

The impact of co-existing TBI and PTSD on symptom severity relative to each condition remains elusive. In one particular study, patients with co-morbid PTSD and mild to severe TBI exhibited fewer intrusive memories than patients with PTSD alone (Hibbard et al. 1998). Another study demonstrated that patients with co-morbid PTSD and moderate to severe TBI report more re-experiencing and hyperarousal symptoms as compared to those with PTSD alone (Simonović, Radisavljević, and Grbeša 2011). Thus there is a heterogeneity and complexity of behavioral phenotypes exhibited by patients with co-morbid PTSD and TBI, which makes it very difficult for clinicians to accurately diagnose.

1. 2 Clinical Diagnosis of PTSD

PTSD involves a constellation of symptoms and is diagnosed clinically based on the criteria outlined in the Diagnostic and Statistical Manual of Mental Disorders, 5th ed. (DSM-5). These criteria include (A) experiencing a traumatic event, (B) intrusions (re-experiencing the traumatic event), (C) avoidance of traumatic cues, (D) negative alterations in cognition and mood and (E) alterations in arousal and reactivity. All criteria must be met, in addition to the persistence of symptoms for more than 30 days following the event (F), for a PTSD diagnosis to be established. If symptoms resolve before this time, the diagnosis is classified as acute stress disorder (American Psychiatric Association 2000).

Patients suspected to have PTSD should be clinically evaluated with a comprehensive psychiatric assessment due to the lack of specific and sensitive clinical biomarkers. Various tools are used by physicians to assist with screening for PTSD and aiding its diagnosis. However, many of these screening tools rely on subjective self-reporting of symptoms. The Primary Care PTSD screening tool is a questionnaire that patients may fill out by themselves or with the aid of a clinician (Prins et al. 2016). The PTSD checklist (PCL-5), is another tool with high sensitivity and specificity that can be used both for screening as well as for monitoring symptom severity and progress (Blevins et al. 2015). With a maximum score of 80, a score of 38 is associated with a diagnosis of PTSD (Spoont et al. 2015). Another assessment tool is the Clinician-Administered PTSD Scale (CAPS). The CAPS involves a structured interview, in which the clinician reads prompts verbatim and assigns a pre-defined symptom severity rating based on symptom frequency and intensity. The CAPS is available in different forms, which address the

assessment of PTSD symptoms occurring in the past week, month, or throughout the lifetime (Nader et al. 1996). The current CAPS (CAPS-5) consists of 30 questions that assess the frequency and intensity of PTSD symptoms. A patient must fulfil the requirements in both frequency and intensity score for each item in order to meet the criteria for a symptom. Table 1.1 depicts the CAPS-5 scoring criteria. A patient must meet these criteria to receive a PTSD diagnosis (Weathers et al. 2013).

Criterion	Condition
A	Has to be met (experiencing a traumatic event)
B	At least one symptom
C	At least one symptom
D	At least two symptoms
E	At least two symptoms
F	Has to be met (disturbance has lasted one month)
G	Has to be met (disturbance causes either clinically significant distress or functional impairment)

Table 1.1. CAPS scoring. Adapted from Weathers et al. 2013.

1. 3 Clinical Diagnosis of TBI

TBI is caused by a forceful blow or impact to the head that is often accompanied by both short-term and long-term neuropsychological symptoms such as confusion, irritability, impulsiveness, and depression (Simonović et al. 2011). Due to the heterogeneous nature of traumatic brain injuries, they can be categorized in several ways.

Most commonly, the Glasgow Coma Scale (GCS) (**Table 1.2**) is used to classify the severity of TBI based on assessment and grading of patient eye-opening, verbal and motor responses. Higher scores are associated with mild injury, whereas lower scores with a more severe injury. While GCS offers a simple and reproducible method, it can also be easily influenced by medical interventions (sedation, intubation, etc.), intoxication (e.g. EtOH or recreational drug use), as well as patient's physical limitations (e.g. paralysis) (Prins et al. 2016). In patients where these limitations are suspected to interfere with the validity of the GCS score, the Full Outline of UnResponsiveness (FOUR) Score may be used. The FOUR Score is based primarily upon neurological examination of the brainstem. Although the FOUR score platform is limited by the greater complexity of the exam as well as the lack of long-term use when compared to the GCS, it has similar sensitivity and specificity for prognosis (Iyer et al. 2009). Low scores are associated with greater hospital mortality according to one study (Wijdicks et al. 2015). In addition to these clinical scores, neuroimaging may also be obtained to assess more severe TBI cases especially when underlying pathologic injuries are suspected. Computed tomography (CT) is the imaging modality of choice in the acute setting. Other neuroimaging techniques such as magnetic resonance imaging (MRI), functional MRI, single-photon emission computed tomography (SPECT) and diffusion tensor imaging (DTI) can also be used to investigate the structural and functional pathological mechanisms (Lee and Newberg 2005). However, limited data is available on the value of the advanced imaging techniques (functional MRI, SPECT and DTI) in routine clinical use at the patient level and therefore are more restricted to use in clinical research (Wintermark et al. 2015). Because of the low detection sensitivity and high cost, conventional imaging techniques (such as CTs and MRI) are rarely used to diagnose mTBI (Lee and Newberg 2005).

Criterion	Response	Score
Eye Opening	Spontaneous	4
	To sound	3
	To pressure	2
	None	1
Best verbal response	Oriented/Normal conversation	5
	Confused/disoriented Conversation	4
	Words	3
	Sounds	2
	None	1
Best motor response	Obeys commands	6
	Localizing response to pain	5
	Normal flexion to pain	4
	Abnormal flexion	3
	Extension	2
	None	1
Total		

Table 1.2. Glasgow Coma Scale

1. 4 Current treatment options for PTSD

Management of PTSD patients relies on several partially effective treatment strategies, which include the use of psychotherapy, such as cognitive behavioral therapy (CBT), sleep modulation, lifestyle interventions and/or pharmacotherapy. These pharmacological interventions mainly rely on few antidepressant drugs, such as selective serotonin reuptake inhibitors (SSRIs) and serotonin noradrenaline reuptake inhibitors (SNRIs) (Tanev et al. 2014). Despite some of the beneficial effects reported using some of these therapies, in most cases patients are typically refractory to treatment (Krystal et al. 2017). The effectiveness of SSRI in treating PTSD was reported to be marginal and comparable to placebo (Ragen et al. 2015). Moreover, in those patients who initially show signs of improvement, there is a high incidence of relapse of clinical symptoms after many

months (Bernardy & Friedman, 2017). Together these underscore the vital need to develop new perspectives regarding the biological responses to PTSD and to identify new and novel therapeutic approaches.

1. 5 Pathophysiology of PTSD

PTSD is a complex disorder that involves multiple system dysregulation. Patients show reduced hippocampal volume, decreased responses of the prefrontal cortex and increased amygdala activity (Fragkaki, Thomaes and Sijbrandij 2016; Sherin and Nemeroff 2011). In addition to these structural and activity changes in the brain, the involvement of the hypothalamic-pituitary-adrenal (HPA) axis, brain-derived neurotrophic factor (BDNF) and neurotransmitters such as glutamate and gamma-aminobutyric acid (GABA) have also been routinely reported. A survey among experts in the field of PTSD research has suggested that the glutamatergic system and HPA axis are among the most promising targets for PTSD treatment and thus will be discussed in more detail in the following sections (Krystal et al. 2017).

Hypothalamic-pituitary-adrenal axis dysregulation

The physiological response to an acute stressor or danger involves the activation of the HPA-axis and the immediate activation of the autonomic nervous system (Mifsud and Reul 2016). The HPA axis (**Figure 1.1**) receives excitatory signals from the amygdala and inhibitory signals from the hippocampus. In the hypothalamus, the paraventricular nucleus responds to stress by releasing corticotropin releasing hormone (CRH), which acts on the

anterior pituitary triggering the release of adrenocorticotrophic hormone (ACTH) into the bloodstream. ACTH stimulates the adrenal cortex to synthesize and release the glucocorticoids cortisol (humans) or corticosterone (rodents). Binding of glucocorticoid to its central receptors in the hippocampus, hypothalamus, and pituitary prevents excess activation of the HPA axis (which constitutes the negative feedback system; Steven E. Hyman 2009).

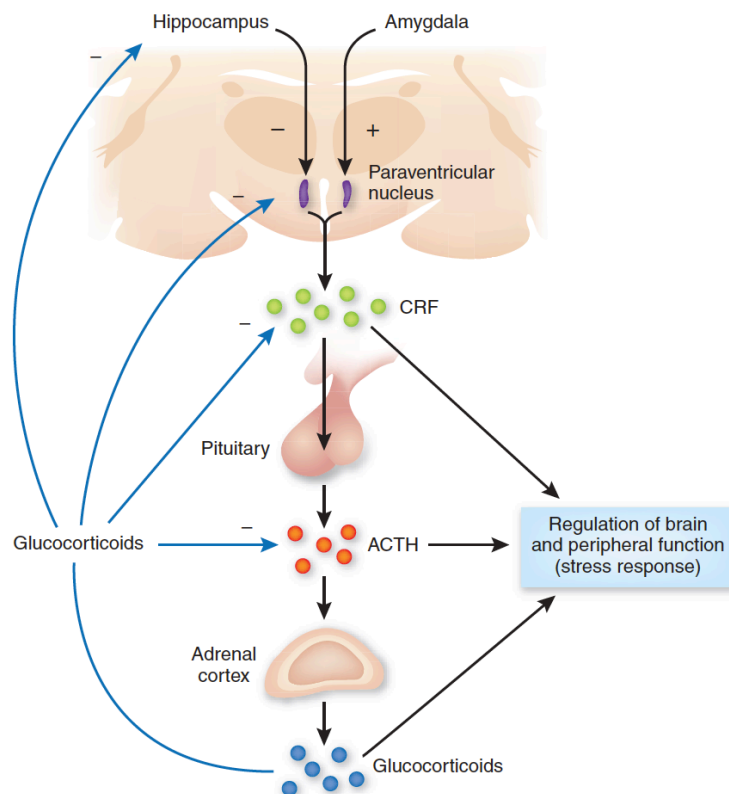


Figure 1.1. The HPA axis. After exposure to stress, the PVN of the hypothalamus releases corticotropin-releasing factor (CRF) which acts on the anterior pituitary to release ACTH into the bloodstream. ACTH will then stimulate the adrenal cortex to synthesize and release glucocorticoids to regulate peripheral and central functions in response to stress. Reprinted from “How adversity gets under the skin”(Hyman 2009). Copyright 2018 by Springer Nature. Reprinted with permission.

Dysregulation of HPA axis feedback mechanisms is implicated in the pathogenesis of PTSD (Leistner and Menke 2018). Several studies have reported lower baseline cortisol levels in PTSD patients, (Yehuda 2001), albeit there is some disagreement, with some reports showing no changes in cortisol levels (Meewisse et al. 2007). The discrepancies between studies may be attributed to differences in sample collection procedures (such as sample type and time of the day in which samples were obtained), the ratio of female to male subjects in patient cohorts, and the type of trauma examined (Matthew J. Girgenti et al. 2017). Moreover, PTSD patients also show increased sensitivity to dexamethasone suppression (Yehuda et al. 1993), which has led to the suggestion that glucocorticoid negative feedback sensitivity is enhanced in PTSD (Yehuda et al. 1993, 1995). As dexamethasone acts mainly on glucocorticoid receptor (GR) in the pituitary, these findings may indicate compromised pituitary function in PTSD. Additional evidence pointing towards impaired glucocorticoid negative feedback regulation in PTSD is the decreased expression of GR co-chaperone FK506 binding protein 5 (FKBP5) in red blood cells and the prefrontal cortex (Averill et al. 2017; Yehuda et al. 2009).

GR located in the in the hippocampus, hypothalamus, and pituitary is the main regulator of glucocorticoids negative feedback (Abdallah, Southwick, and Krystal 2017; Hyman 2009). In the absence of stress (normal glucocorticoid levels), GR is mainly localized in the cytoplasm in an inactive complex containing co-chaperones heat shock protein 90 (HSP90) and FKBP5. After HPA axis activation, plasma glucocorticoid levels increase, dissociate from the bound plasma proteins and passively diffuse across plasma membranes to bind glucocorticoid receptor in the cytoplasm. Glucocorticoids can diffuse easily through the blood-brain barrier (BBB) because of their small size and lipid solubility. Binding of glucocorticoids to the GR complex results in the dissociation of

FKBP5 and HSP90 allowing the activation and translocation of GR to the nucleus (**Figure 1.2**). Once in the nucleus, GR binds to glucocorticoid response elements (GRE) initiating gene expression of a number of proteins such as FKBP5 and CRH (**Figure 1.2**) (Matthew J. Girgenti et al. 2017). Because FKBP5 bound GR is inactive in the cytoplasm, low levels of cytoplasmic FKBP5 would result in decreased inhibitory control of GR and enhanced GR sensitivity. Enhanced GR sensitivity would subsequently result in a reduction in basal glucocorticoid levels. Alternatively, it was suggested that the change in FKBP5 levels is an adaptation to low cortisol levels in PTSD subjects rather than being a risk factor or a cause of HPA axis dysregulation (**Figure 1.2**).

Interestingly, FKBP5 genotype has been reported to contribute to risk for PTSD. The interaction between polymorphisms in the FKBP5 gene and exposure to child abuse could predict the severity of PTSD symptoms in adult life (Binder et al. 2008; Watkins et al. 2016). In particular, the FKBP5 rs9470080 genotype was shown to moderate that risk (Xie et al. 2010). In addition, early trauma victims who carry the risk allele showed decreased DNA methylation in intron 7 of FKBP5 leading to increased gene expression and glucocorticoid receptor resistance (Klengel et al. 2013).

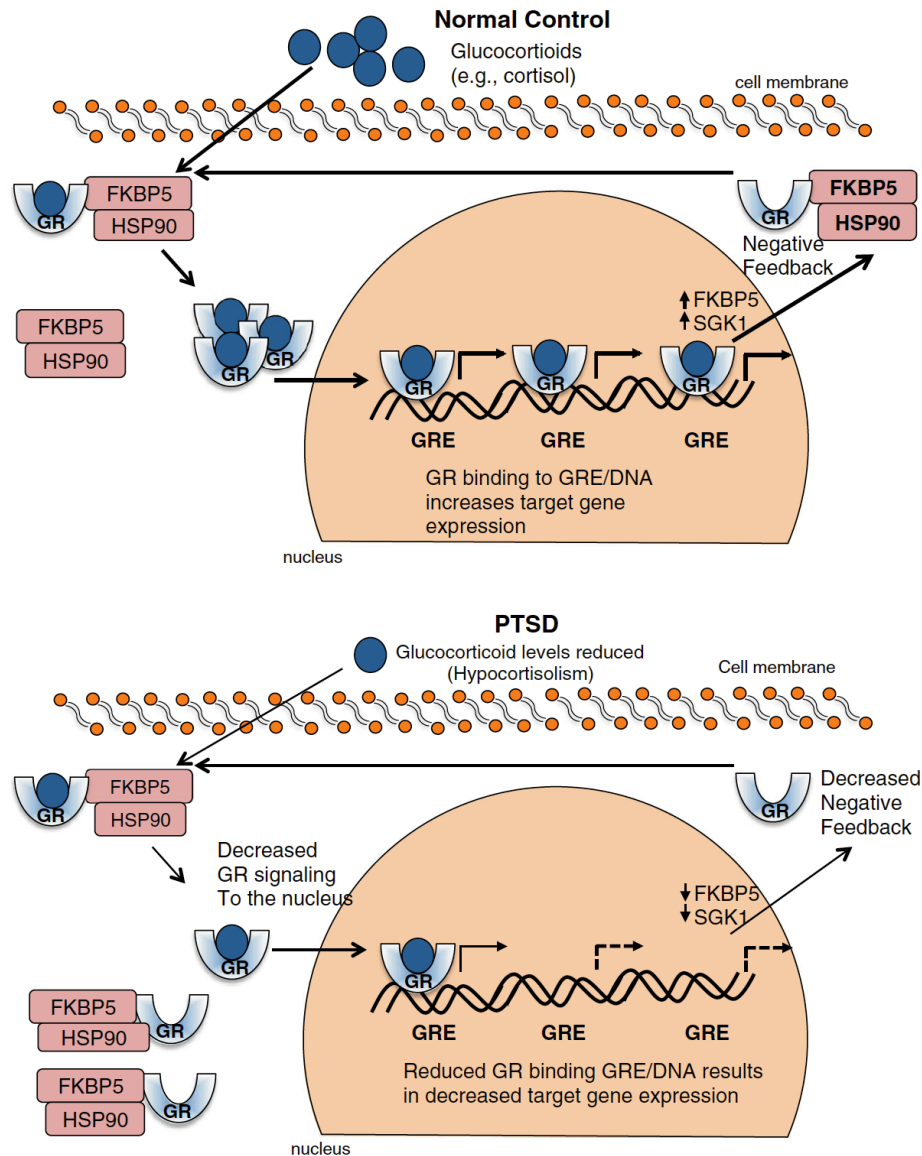


Figure 1.2. A hypothesized mechanism for the regulation of glucocorticoid signaling in PTSD. After HPA axis activation, cytoplasmic GR binds to glucocorticoids (e.g. cortisol) and translocates to the nucleus increasing gene expression of target genes such as FKBP5. Increased FKBP5 expression creates an ultra-short intracellular feedback loop that limits excessive GR activation. The chronic decrease in baseline cortisol levels in PTSD patients may result in decreased GR activation as an adaptive mechanism which in turns lead to decreased expression of GR target genes such as FKBP5. Reprinted from “Molecular and Cellular Effects of Traumatic Stress: Implications for PTSD” (Matthew J. Girgenti et al. 2017). Copyright 2018 by Springer Nature. Reprinted with permission.

GABAergic and glutamatergic abnormalities

Disturbances in GABA and glutamate levels in PTSD patients have been examined in several human studies. The majority of studies report a decrease in GABA levels in the serum and in multiple cortical regions (Geuze et al. 2008; Pennington et al. 2014; Rosso et al. 2014), and an increase in serum glutamate levels (Nishi et al. 2015). Some contradictory findings have also been reported (Averill et al. 2017). Additionally, multiple preclinical and clinical studies have demonstrated the potential of ketamine (a glutamate N-methyl-D-aspartate (NMDA) receptor antagonist) in the treatment of mood and anxiety disorders (Costi, Van Dam, and Murrough 2015). In a randomized clinical trial, ketamine was shown to be effective in the rapid reduction of symptom severity in chronic PTSD patients (Feder et al. 2014). **Figure 1.3** depicts a proposed mechanism for the role of GABA and glutamate system in mediating PTSD pathophysiology.

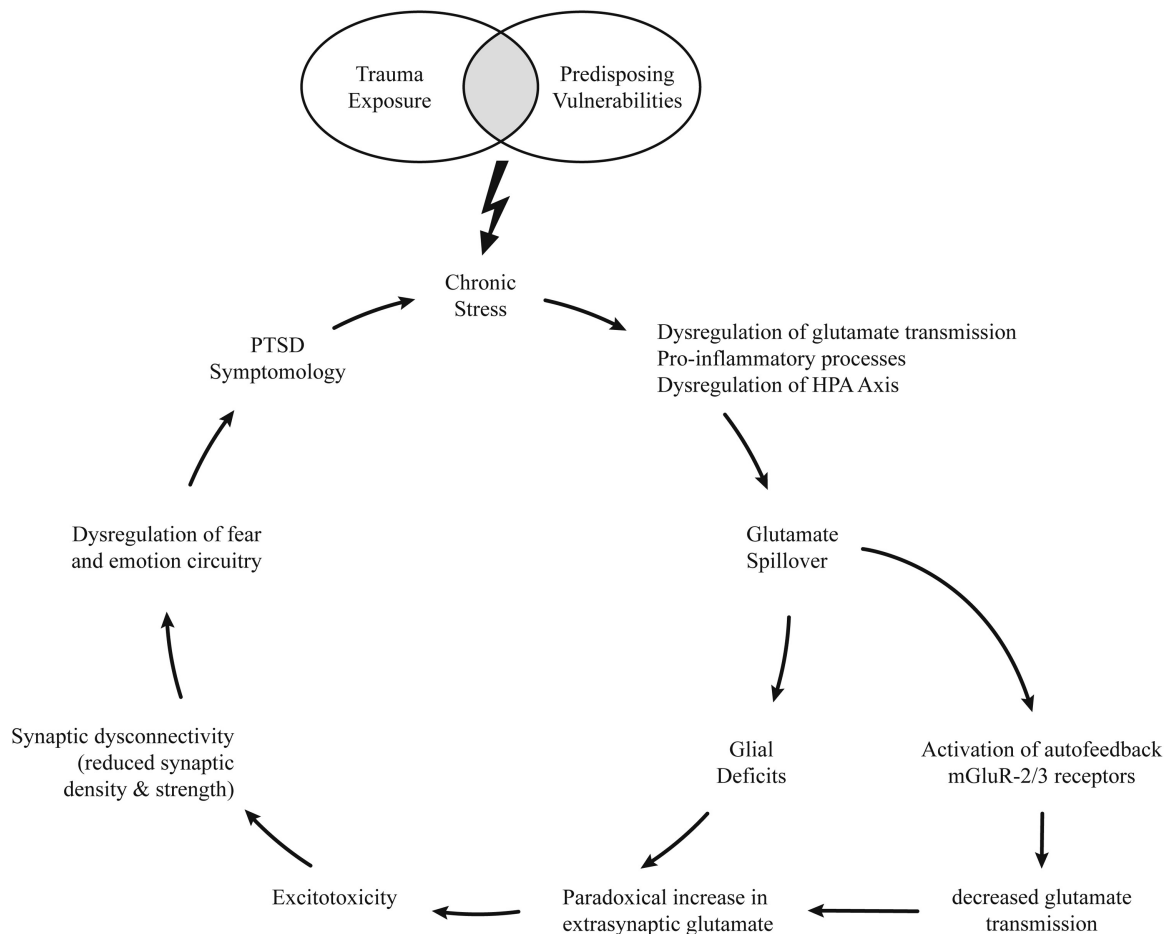


Figure 1.3. A schematic for the proposed role of GABAergic and glutamatergic abnormalities in PTSD pathophysiology. The interaction between predisposing vulnerabilities (risk factors) and traumatic stress results in a dysregulation in glutamatergic signaling, inflammatory processes and the HPA axis ultimately leading to increased brain glutamate levels and excitotoxicity. These events are followed by synaptic dysconnectivity in brain regions responsible for emotional regulation and stress response and the subsequent emergence of some PTSD symptoms. Reprinted from “Glutamate Dysregulation and Glutamatergic Therapeutics for PTSD: Evidence from Human Studies”(Averill et al. 2017). Copyright 2018 by Elsevier. Reprinted with permission.

Alterations in Brain-Derived Neurotrophic Factor

BDNF is a member of the neurotrophin family of nerve growth factors. The mature form of BDNF influences several fundamental brain functions including neuronal survival, growth and synaptic plasticity (Phillips 2017).

PTSD patients show lower serum BDNF levels (Angelucci et al. 2014) relative to non-traumatized control subjects. In animal studies, exposure to acute or chronic stress is associated with a decrease in hippocampal BDNF levels in the hippocampus (Lee and Kim 2010; Yu and Chen 2011).

A BDNF gene polymorphism has been reported to confer risk for a number of psychiatric disorders including PTSD. A single nucleotide polymorphism (SNP) in the BDNF gene resulting in a valine to methionine substitution (V66M) is associated with reduced hippocampal volume (Brooks et al. 2014) and altered fear extinction and hyperarousal symptoms in PTSD subjects (Soliman et al. 2010; Zhang, Li, and Hu 2016). In animal studies, V66M was also associated with increased anxiety-related behaviors (Chen et al. 2007). Furthermore, long-term epigenetic changes in hippocampal BDNF DNA were reported after chronic psychosocial stress in rats (Roth et al. 2011).

1. 6 Pathophysiology of TBI

Primary and secondary injuries occur after sustaining a TBI. Primary injuries are the result of direct physical or mechanical damage at the initial moment of impact. Secondary injuries are a number of delayed pathological and biochemical disturbances that occur over the course of hours to months after injury (Marklund and Hillered 2011). The cascade of secondary injuries is initiated by an increase in the release of excitatory neurotransmitters such as glutamate and aspartate, which in turn activate glutamate receptors and increase Ca^{+2} influx. The increased influx of Ca^{+2} results in increased production of reactive oxygen species, mitochondrial damage, an increase in the release of chemokines and cytokines, and changes in gene expression. These cytotoxic events will eventually lead to blood-brain barrier damage, cell death, axonal injury and impaired cognitive functions (Xiong, Mahmood, and Chopp 2013). Changes in synaptic plasticity are also implicated in TBI-related cognitive dysfunction especially in mild to moderate cases where cell death and neuronal degeneration are not obvious (Walker and Tesco 2013). The goals of TBI therapeutic interventions are to prevent or alleviate secondary injuries, since primary injuries can be only prevented by taking precautionary measures (Marklund and Hillered 2011).

The majority of patients who endure a single concussion (mild TBI) will have a complete recovery after receiving proper management (Kamins and Giza 2016). One of the main aims of care in concussion patients is to decrease the risk of exposure to a second concussion, since multiple concussions are well known to increase symptom severity and prolong recovery times (Barkhoudarian, Hovda, and Giza 2011). Recently, the consequences of enduring multiple concussions in athletes and military personnel have

received a lot of attention because of its implications in the development of Chronic Traumatic Encephalopathy or CTE (Omalu et al. 2011). CTE is a neurodegenerative disease characterized by the accumulation of hyperphosphorylated Tau protein in different brain regions, and has been associated with severe alterations in mood, behavior and cognition (McKee et al. 2015; Omalu et al. 2011).

1. 7 Preclinical animal models for PTSD

Although the impact of neuropsychiatric disorders on public health is enormous, the progress in understanding their pathophysiology, and consequently developing new treatment strategies, has been very slow and yielded very little success. The ethical and technical challenges surrounding examining the living human brain are likely responsible for the lack of advancement (Nestler and Hyman 2010). Heterogeneity of trauma type and exposure, lack of prospective design studies and available autopsy tissue material also preclude our ability to adequately interrogate the pathophysiological mechanisms in humans. Animal models provide an alternative platform to conduct more invasive and controlled longitudinal studies in response to different stressors, but to be of value such a model must be ethologically valid and demonstrate relevance to the human condition. Several criteria have to be established when assessing the validity of animals models including: (1) *Face validity* which assesses the similarities between the symptoms produced by the animal model and the human condition, (2) *Construct validity* which accounts for the accuracy of the model to measure what it was designed for, and (3) *Predictive validity* which examines the ability of the model to predict treatment outcomes (Belzung and Lemoine 2011; Goswami et al. 2013).

Modeling PTSD in animals is complex, given the limitations in our ability to model and assess certain neurobehavioral human patterns in animals such as guilt and shame. However, most studies attempt to recapitulate some behavioral traits similar to the cluster of symptoms experienced in human PTSD patients as defined by the DSM-5 criteria, such as anxiety, startle response, deficits in social interaction, depression, avoidance-like behavior, reduced exploration and reconsolidation of traumatic memories (American Psychiatric Association, 2000). Typically, these PTSD-like symptoms are induced in animals by exposing them to one or more aversive stimuli for variable durations to establish the face validity of the model (Bowers and Ressler 2015; Matar et al. 2013; Sambasivarao 2013).

Summary of the most widely used stress procedures to model PTSD in Animals

Stressors used in PTSD animal models can be broadly divided into three categories; 1) physical such as inescapable foot-shocks, forced swim, and restraint stress, 2) social such as unstable housing, social isolation, maternal separation and social defeat, and 3) psychological stressors involving physical exposure to a predator or exposure to a predator odor (Blanchard and Caroline Blanchard 1977; Matar, Zohar, and Cohen 2013; Sambasivarao 2013). Most of these stressors have been used alone or in combination to produce PTSD-like behavioral and neurobiological changes. The challenge, however, in developing an ethologically relevant animal model of PTSD lies in the ability to demonstrate face, construct, and predictive validity (Goswami et al. 2013; Matar et al. 2013). Most preclinical studies to date have not been able to fully satisfy all these combinatory criteria, perhaps most important of which is the persistence of symptoms at

chronic time points (many months) after trauma; thus, more studies need to be conducted in vivo to improve on currently available animal models. A brief summary of the most widely used PTSD animal models (**Figure 1.4**) will be presented in the following section.

Social defeat

Social defeat is a well-established model for depression and PTSD (Blanchard and Caroline Blanchard 1977). In this model, test mice can interact with an aggressive dominant mouse, typically of a different genetic background (example CD1 or BALB/c mice if the test mouse is C57BL/6), and they are subsequently transferred to a cage that allows for olfactory and auditory but not visual communication (**Figure 1.4F**). The auditory and olfactory communication is used as a reminder of the defeat sessions (Hollis and Kabbaj 2014). Animals in this model can then be tested for social dominance by placing them in a box that contains two cages, one empty and one containing the aggressive mouse. Defeated mice will tend to spend more time around the empty cage (Brachman et al. 2016). This social avoidance behavior has been reported 10 days after the social defeat procedures (Toth and Neumann 2013). Additional behavioral changes such as hyperarousal and anhedonia (decreased sucrose preference) have also been reported at around 3 weeks after the last stress (Wood et al. 2015). Mice exposed to the social defeat paradigm typically show increased baseline plasma corticosterone at 12 days after stress (Page, Opp, and Kozachik 2016).

Response to predator stress

In the predator stress model, animals receive single or multiple exposures to a physical predator or a predator scent such as cat feces, fox urine or their components (**Figure 1.4B**). Studies have shown anxiety-like behavior and freezing in response to trauma reminders (3 months after predator stress) (Zoladz et al. 2015). The impact of predator stress on other behavioral domains such as anhedonia and stress-coping is not clear (Deslauriers et al. 2018). While elevated baseline plasma corticosterone levels are observed at 7 days after exposure to predator scent (Cohen et al. 2010), low baseline plasma corticosterone levels were reported in the predator based psychosocial stress model (PPS) involving exposure to a predator and daily unstable social housing (Zoladz, Fleshner, and Diamond 2012). The PPS model also shows increased sensitivity to dexamethasone suppression in stressed rats; a finding that has been documented in multiple studies of PTSD patients (de Kloet et al. 2007; Nijdam et al. 2015; Yehuda 2001; Yehuda et al. 1993).

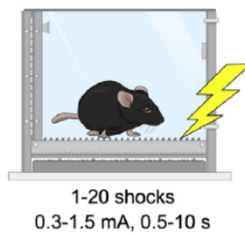
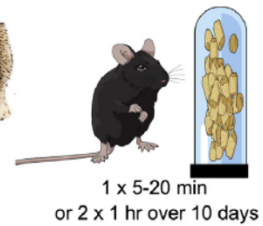
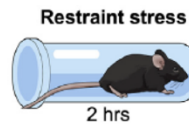
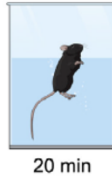
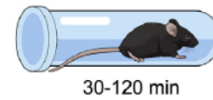
Single prolonged stress

Single prolonged stress (SPS) relies on a single episode of intense stressful events to trigger several PTSD-related phenotypes (Liberzon, Krstov and Young 1997). The procedures of SPS are quite standard and involves a 2-hour immobilization or restraint, followed by 20 minutes of forced swimming, and finally exposure to diethyl ether for 2 to 3 minutes (**Figure 1.4C**). The SPS model shows a number of behavioral deficits such as passive stress-coping, anxiety-like and impaired fear extinction 7-21 days after the last stress (Borghans and Homberg 2015; Deslauriers et al. 2018). A key limitation to the SPS

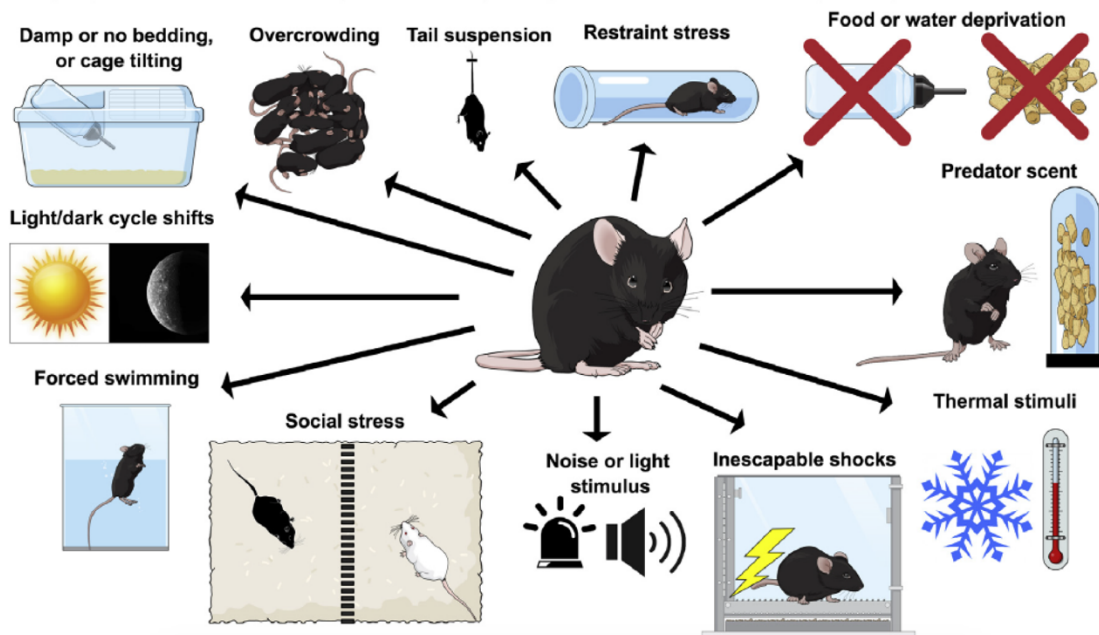
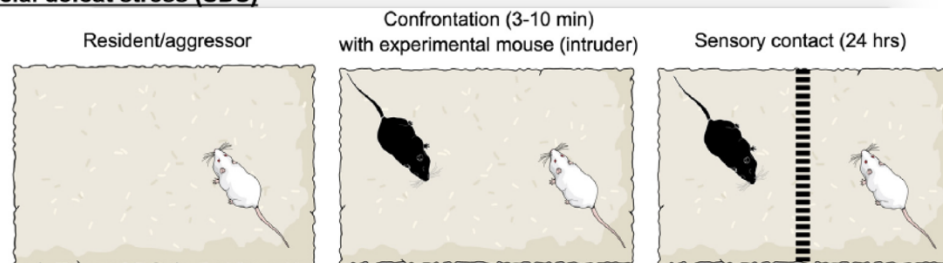
model is that baseline corticosterone levels are elevated at day 7 after exposure (Deslauriers et al. 2018; Kohda et al. 2007). However, the model is successful in demonstrating enhanced glucocorticoid negative feedback possibly due to overexpression of GR in brain regions mediating activity of the HPA-axis during stress (Yamamoto et al. 2009).

Unpredictable variable stress

Unpredictable variable stress (UVS) or chronic unpredictable stress (CUS) is generally considered a model of depression, since it involves prolonged exposure to mild to moderate stressors. Prolonged exposure to life-stress is implicated in stress-induced depression in contrast to PTSD which usually emerges from a sudden intense exposure to a single trauma. However, it is argued that the repetitive nature of this paradigm is relevant to combat-related PTSD where the exposure to trauma is recurrent and prolonged (Richter-Levin et al., 2018). In this paradigm, animals are exposed to multiple stressors over the period of 4 to 8 weeks in an unpredictable manner (Monteiro et al. 2015). The stressors used in this paradigm can vary in intensity between paradigms as depicted in **Figure 1.4E**. Changes in body weight, avoidance and stress-coping have been robustly reported at the end of the stress procedures (Monteiro et al. 2015). However, few studies have investigated the persistence of behavioral deficits after CUS cessation possibly because of its common use as a stress-induced depression models where it is suitable for the stress procedures to be ongoing. CUS mice have been demonstrated to show elevated baseline corticosterone levels at 1-7 days after the last stressor (Cordner and Tamashiro 2016).

A Inescapable shocks**B**Unprotected exposurePredator stressProtected exposurePredator scent**C Single prolonged stress (SPS)**Forced swimming**D Immobilization (IMO) or restraint stress****E Unpredictable variable stress (UVS)**

Daily exposures to various stressors (chosen randomly among the stressors described below) over a period of 1 to 8 weeks

**F Social defeat stress (SDS)**

The paradigm is repeated for typically 5 to 21 consecutive days.

Figure 1.4. A number of the most widely used PTSD animal models. In fear conditioning experiments (A), animals are exposed to inescapable footshocks that are often paired with a cue or reminder. Predator stress models involve single or multiple exposures to a predator (e.g. cat) or a predator scent (e.g. fox urine) (B). Panel (C) depicts the three consecutive stressors implemented in the single prolonged stress model. Restraint or immobilization stress procedures (D). Examples for the stressors used in unpredictable variable stress (UVS) or chronic unpredictable stress (CUS) models are depicted in panel (E). The social defeat model involving multiple encounters with a dominant aggressive mouse is depicted in panel (F). Reprinted from “Current Status of Animal Models of Posttraumatic Stress Disorder: Behavioral and Biological Phenotypes, and Future Challenges in Improving Translation”(Deslauriers et al. 2018). Copyright 2018 by Elsevier. Reprinted with permission.

Experimental approaches to evaluate PTSD-related behavior in animal models.

The main experimental tools used to test the *Face validity* of animal behavior in PTSD models are briefly summarized in the following section.

Fear Conditioning

Pavlovian fear conditioning is among the methods commonly used to evaluate abnormal fear (Maren 2001). Briefly, animals receive an unconditioned stimulus (aversive stimulus) paired with a conditioned stimulus (a tone) in a particular context. The conditioned stimulus serves as a predictor of traumatic aversive stimulus. Animals are then tested for freezing behavior (a measure of fear) in the same (or novel) context with or without the conditioned stimulus, determining “cued” or “contextual” fear responses. Animals with intensified fear will show greater freezing behavior even in the absence of aversive stimuli (Bowers and Ressler 2015). Preclinical models using foot-shocks of various intensities, frequency and duration are routinely applied to investigate the underlying neurobiology of adaptive and pathological fear (Bowers and Ressler 2015).

Social behavior

Alterations in social functioning (e.g. social anxiety and avoidance and occupational impairments) have been noted in PTSD patients (Frueh et al. 2001). Social behavior can be investigated in animals using the three-chamber test, which evaluates the time spent by a mouse interacting between an empty chamber and a chamber holding a new novel stranger mouse (Egashira et al. 2007). Animals exposed to trauma, with impaired social behavior, tend to spend less time interacting with a new novel mouse compared to their control counterparts (Egashira et al. 2007).

Anxiety-like behavior

Anxiety is another PTSD-related behavior that is frequently evaluated in animal models (Tanev et al. 2014). The Elevated Plus Maze (EPM) is a traditional test used to assess anxiety in animal models (Bailey and Crawley 2009; Handley and Mithani 1984). The apparatus consists of two open and two closed arms forming a plus shape. Normal animals tend to spend a proportion of time exploring the open arms of the maze while animals with symptoms of anxiety tend to spend most of their time in the closed arms. On the contrary, animals with impulsive-like behavior (disinhibition) tend to spend more time exploring the open arms (Bortolato et al. 2009). The Open Field is another routinely used behavioral test used to assess anxiety and general locomotor activity (Hall and Ballachey 1932). The test field is a simple enclosure (circular, rectangular or, square) surrounded by walls to prevent escape. Healthy animals will typically spend time exploring the border of the field in contact with the walls but will also spend a percentage of their time exploring

the unshielded center zone. Spending significantly less time exploring the center zone is used as an index of anxiety (thigmotaxis), while spending relatively more time exploring the center zone implies increased exploratory behavior and/or disinhibition (Bailey and Crawley 2009).

Stress-coping behavior

The Forced Swim Test is usually implemented to assess stress-coping behavior. The test procedures involve placing the animal for 6 min in a cylindrical vessel (20cm X 20 cm) containing water at room temperature. In response to the forced swim stress, animals either swim, float, or struggle (climb the walls of the container). The time spent swimming and struggling is interpreted as positive stress-coping strategy, while the time spent floating (immobile) is interpreted as a passive stress-coping strategy. Antidepressant medications are successful in decreasing rodents' immobility time in the FST, therefore the passive stress-coping behavior is often referred to as depression-like behavior in the literature (Brachman et al. 2016). However, recent articles have considered the "stress-coping strategy" terminology to be more accurate in describing the behaviors assessed by the FST (Commons et al. 2017; Molendijk and de Kloet 2015).

1. 8 Preclinical animal models of mTBI.

Because of TBI heterogeneity and the ethical and technical limitations of human studies noted above, preclinical animal models provide a valuable platform to examine the pathobiological mechanism of TBI in a focused and well-controlled setting (Xiong et al. 2013). Several procedures have been developed to model different types and severities of TBI in rodents. In the context of mild TBI, closed head injury models (such as the weight-drop and pneumatic impactor models) and blast injury models are often used (Shultz et al. 2017).

Our group has ample experience in developing single and repetitive mTBI mouse models that have been characterized at 24 hours and up to 24 months after injury. In agreement with clinical findings in concussion patients, we have shown that exposure to repetitive mTBI results in progressive cognitive deficits and behavioral impairments at 12 months after the last injury (Mouzon et al. 2014, 2012; Ojo et al. 2013). These impairments were absent after a single mTBI. In addition, the behavioral alterations after repetitive TBI (5 hits with intra-hit 48-hours interval) were accompanied by progressive neuroinflammation and persistent white matter degeneration (Mouzon et al. 2014). Furthermore, increasing the number of hits to 24-32 hits with 48-hours interval resulted in the accumulation of hyperphosphorylated tau and cognitive deficits in mice expressing humanized tau 3 months post-injury (Ojo et al. 2016).

1. 9 Summary of preclinical models for PTSD and co-occurring TBI.

Very few studies have investigated the effects of both mTBI and PTSD in animal models. A study by Kwon and colleagues, supporting a synergistic relationship between TBI and traumatic stress, investigated the long-term consequences of unpredictable stress in combination with a blast injury. These studies reported impairment in memory function in blast injured mice exposed to chronic and unpredictable predator odor (Kwon et al. 2011). In another study, Klemenhausen et al. found that repetitive concussive traumatic brain injury and post-injury foot shocks had synergistic effects in tests of social recognition and depression-like behavior, compared to non-shocked injured mice or shocked but uninjured mice that were less impaired (Klemenhausen, O'Brien, and Brody 2013). On the other hand, some recent studies have reported that mTBI may mitigate the PTSD-related behavior. Genovese et al. studied the effect of mTBI from a blast overpressure (BOP) on the expression of a conditioned fear in rats. Interestingly, BOP injuries decreased the expression of a conditioned fear response in rats exposed to inescapable electric shocks relative to rats receiving shocks only (Genovese et al. 2013). A study from our laboratory has found that mTBI abrogates both contextual fear and impairments in social behavior, seen in PTSD animals (Ojo et al. 2014). Finally, a study by Sierra-Mercado et al. has shown that controlled cortical impact (CCI) injury had no influence on fear learning and extinction irrespective of when it was applied before or after fear conditioning (Sierra-Mercado et al. 2015)

A different topic that has been more extensively examined in the literature is the risk of developing PTSD-like symptoms after mTBI alone in animals. Six weeks after mTBI in rats, Elder et al. found a decrease in open-field center activity following exposure to

a predator cue and a decrease in the exploratory activity in an elevated plus maze (Elder et al. 2012). These changes, together with increased acoustic startle response (a rapid and involuntary muscle reflex to auditory stimuli), and conditioned fear were interpreted as PTSD-like traits (Elder et al. 2012). Another study by Reger et al. reported an overall increase in fear conditioning in rats several days after a fluid percussion injury (Reger et al. 2012). Similarly, Meyer et al. observed an increase in conditioned fear (8 days post-injury) and a decrease in open-arm time in a plus maze after a weight drop injury (Meyer et al. 2012).

1. 10 Hypotheses and Synopsis of the following chapters

Developing a preclinical model that captures the complexity of both PTSD and r-mTBI, either individually or comorbidly, is crucial to understand the underlying pathophysiology and to identify targets for therapeutic intervention. However, as presented in this Introduction, there are innumerable potential paradigms that could be conceived to model PTSD and PTSD with comorbid TBI. While these paradigms have been successful in probing several acute PTSD-related or mTBI-related phenotypes, studies exploring the long-lasting behavioral effects in correlation with the key biological outcomes of PTSD and/or TBI are lacking. When designing a new model, it is important to recapitulate multiple symptoms and pathologies in a manner and timescale that will be relevant to the human population. In addition, these models have limitations in modeling specific phenotypes such as HPA axis dysregulation in PTSD. For instance, CUS and SPS paradigms often result in an increase in basal plasma glucocorticoid levels after stress

cessation, while they appear to be low or unchanged in PTSD patients (Deslauriers et al. 2018; Yehuda 2001).

Our group has previously developed a mouse model of PTSD with comorbid TBI, which unlike other models that examine the effects of TBI and traumatic stress at different timepoints, couples both events at around the same time. This approach is relevant to a large population of PTSD patients (such as the combat veteran population) who experience an emotional trauma around the same time that they also experience TBI. In our previous model, a combination of unpredictable exposure to psychological stressors (trimethylthiazoline—TMT, a component of fox urine), a physical stressor (one foot-shock), and one mTBI were employed. This paradigm resulted in distinct and overlapping effects in neurobehavioral, neuropathological and biochemical profiles subacutely at 14 days after exposures (Ojo et al. 2014). However, these effects did not persist at a chronic timepoint. The goal for this new body of research was to overcome the limitations of our previous model and advance towards a valid model both for PTSD and PTSD with coincident TBI that shows long-lasting behavioral and neurobiological alterations. Such a model could then be utilized to advance the understanding of the pathophysiology of the condition, and in accelerating the drug discovery process.

My hypothesis was that with a new chronic stress exposure paradigm that combines physical, social and psychological stressors, I would develop a novel mouse model of PTSD with face and construct validity and a persistent phenotype. After establishing the PTSD model, the addition of our well-characterized r-mTBI protocol to the stress procedures would then show altered outcomes.

In chapter 2, I first focused on establishing a PTSD mouse model that showed chronic stress-related effects at a chronic timepoint. To accomplish that, I used a more intensified stress procedure than that previously employed, including repeated unpredictable stress (RUS) and social isolation. To validate the model, I examined the behavioral outcomes at extended timepoints (acute to chronic). In addition, I explored the impact of the stress procedure on a number of PTSD-related pathobiological mechanisms such as the HPA axis and hippocampal structure and plasticity.

I then examined the effects of repeated exposure to mild TBI in addition to stress in chapter 3, to explore the impact of repetitive mild TBI (r-mTBI) on stress-related phenotypes. The procedures of r-mTBI and RUS will be delivered twice to resemble the multiple traumatic events and injuries that are usually sustained by soldiers receiving multiple deployments and to address a possible dose-response relationship of trauma, TBI and their interaction.

In chapter 4, I specifically interrogated the role of the HPA axis in our stress model using different challenges and molecular tools. Finally, I present the findings of a pilot treatment study in chapter 5, involving supplementing GR signaling using low dose dexamethasone and replenishing BDNF levels using 7,8 dihydroxyflavone (A TrkB receptor agonist), individually or as a combined therapy, in this mouse model of PTSD.

Chapter 2. Chronic hippocampal abnormalities and blunted HPA axis in an animal model of repeated unpredictable stress

2. 1 Introduction

Upon exposure to an acute stressor, the brain initiates “fight or flight” responses involving the cardiovascular, endocrine and immune systems to prepare the body for the possible danger. After dismissal of the stressor, physiological homeostatic mechanisms restore normal baseline functions. However, a single exposure to a particularly severe traumatic stressor or prolonged exposure to milder stressors, can lead to maladaptive responses within the brain neurocircuitry regulating these homeostatic mechanisms (Bonne et al. 2004; Roth et al. 2012; Sambasivarao 2013; Sherin and Nemeroff 2011). These maladaptive responses are involved in the pathophysiology of various stress-related psychiatric illnesses such as anxiety disorders, post-traumatic stress disorder (PTSD) and depression (MCEWEN 2004).

As described in chapter 1, the symptoms of PTSD are marked by the uncontrolled re-experiencing of traumatic events triggered by intrusive memories, avoidance of traumatic cues, negative alterations in cognition and mood and dynamic fluctuation in arousal and reactivity states (DSM-5, 2013) (American Psychiatric Association 2000). A diagnosis of PTSD requires that these clusters of symptoms must last for at least one month to demonstrate clinical impact, and they have to significantly influence the functional ability of the individual in several domains.

It has previously been shown that exposure to a single footshock with repeated exposure to TMT under restraint induces acute PTSD-like behavior including anxiety-like

behavior, social avoidance and recall of fear memory in C57BL/6 mice (Ojo et al. 2014). One of the major limitations of our previous model was the lack of chronic and persistent behavioral and neurobiological changes. The presence of acute symptoms after a traumatic incident is not an accurate hallmark feature for PTSD diagnosis, as they can be attributed to a normal adaptive stress response or acute stress disorder (Flandreau and Toth 2017). While acute stress disorder and PTSD share a number of diagnostic criteria, in PTSD, symptoms are primarily persistent (> 1 month) and may also develop after a delay (American Psychiatric Association 2000). Therefore, a chronic and long-lasting behavioral and biological outcome in a mouse model is essential to more accurately reflect the human PTSD experience.

To overcome this limitation in our earlier model, I intensified our previous 21-day repeated unpredictable stress (RUS) paradigm to include 10 unpredictable exposures to TMT under restraint, 5-inescapable foot shocks and daily unstable social housing that was followed by social isolation. The presence of chronic social stress in our model is a unique feature in comparison with traditional CUS paradigms.

Here, I show that such repeated exposures to intense physical, psychological and psychosocial stressors resulted in PTSD-like symptomology and biological responses as presented in the following sections.

2. 2 Materials and Methods

Animals

C57BL/6 male mice (aged 8–10 weeks) were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed in standard cages under a 12-h light/12-h dark schedule at ambient temperature. A two-weeks habituation period was followed before the start of any experimental procedures. All procedures were performed in accordance with OLAW guidelines under an approved protocol by the Roskamp Institute IACUC.

The 21-day RUS paradigm

Animals were randomly assigned to either a control group (n=15) or the stress group (n=17). The 21-day repeated unpredictable stress (RUS) paradigm is shown in **Figure 2.1**, and involved (i) daily unstable social housing with an alternate congener; (ii) unpredictable repetitive exposure to a psychological stressor of predator odor (TMT) while in a decapicone restrainer for 30 mins in a dark room (1 exposure per day, 10 exposures within 21 days); (iii) physical trauma in the form of five repeated inescapable foot-shocks; and (iv) lack of social support (i.e. singly housed) post-traumatic stress. Animals in the stress group were housed individually after the 21 days, whereas animals in the control group were housed in groups of 2-3 throughout the study. Animals in the control group were never subjected to the decapicone restraint or TMT exposure, but were exposed to the fear-conditioning chamber for the same frequency and duration as the stressed mice, without administration of foot shock or auditory cues. 4-5 animals per group were euthanized at 10 days after RUS (acute timepoint) for molecular studies without receiving any behavioral testing. The remaining animals (10-12) per group received a behavioral test

battery at one day (acute timepoint) after RUS and then were retested using a similar battery at 3 months and 6 months after RUS. Details about the timeline of the behavioral test battery can be found in **Figure 2.2** After the last behavioral experiment, all animals (11-12) were euthanized and brain and plasma were collected for molecular studies (6 months timepoint).

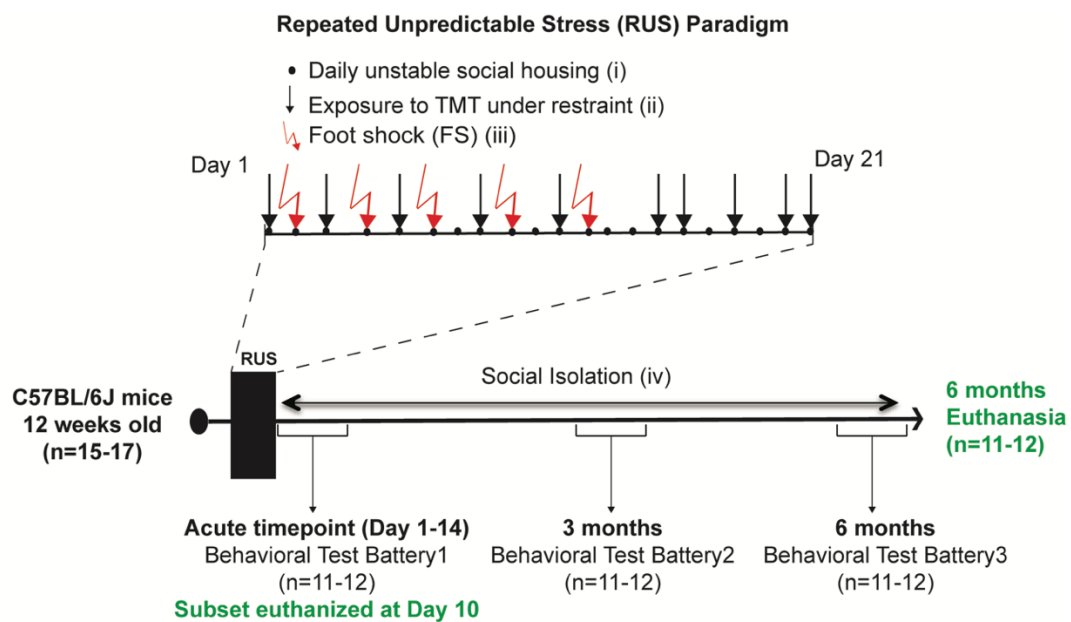


Figure 2.1. Study timeline and experimental procedures for the stress paradigm.

Stress paradigm involved 21 days of daily unstable social housing with an alternate congener (i), unpredictable repetitive exposures to danger-related predator odor (Fox urine, TMT), while under a decapicone restrainer for 30 mins (ii), and physical trauma in the form of five repeated inescapable footshocks (iii). After 21 days of stress, animals in the stress group were singly housed (social isolation) until the end of the study (iv). A battery of behavioral testing was conducted at an acute timepoint (1-14 days), 3 months, and 6 months after the last footshock. Brain tissue and plasma were collected only at the acute timepoint (10 days) and 6 months after the last footshock.

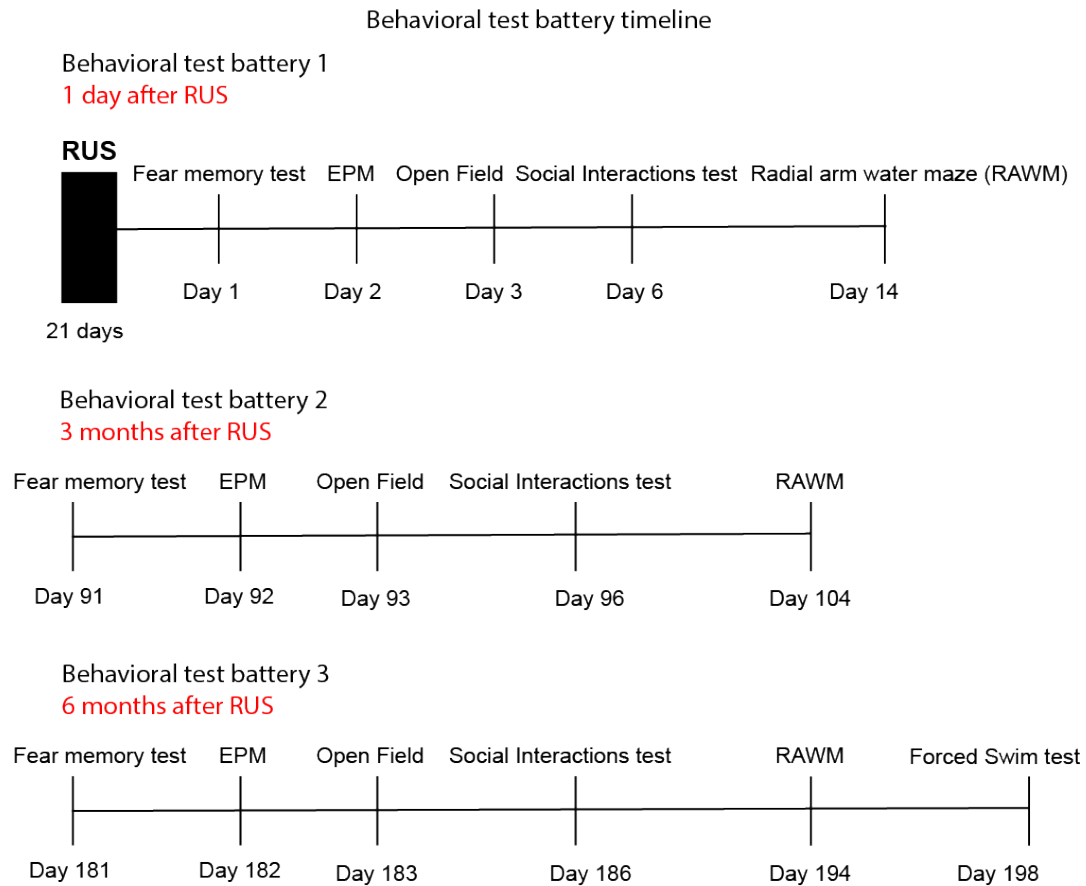


Figure 2.2. Timeline of behavioral test batteries. The same animals tested in behavioral test battery 1 were retested at behavioral test batteries 2 and 3.

TMT

Trimethylthiazoline (TMT, SRQ Bio, Florida) is a component of fox urine, which I and others have previously used as a predator odor stressor (Endres and Fendt 2009; Janitzky et al. 2015). TMT was diluted 1:10 (v/v) in double distilled water before being used as a predator odor stressor. 50 μ l of 10% TMT were dispensed on a tissue paper then placed next to the restrained animal.

Behavioural testing

At least two baseline body weight measurements were taken during the acclimation period and before administering the stress paradigm. After exposure to the stress, body weight was measured on a weekly basis for each mouse.

Fear conditioning, contextual and cued fear testing.

Fear conditioning was delivered in the form of 5 footshocks on days 2, 4, 6, 9, and 12 of the stress exposures paradigm. This involved placing animals in a shuttle box-chamber for 3min, with a pure tone (70 dB, 2.9 kHz) introduced during the last 30 s. At the end of the auditory cue, animals received a single 1mA of uncontrollable/inescapable footshock for 4s and were allowed to recover for 1min in the same context before returning to their home cages. The control group was allowed to explore the fear-conditioning context for 3 min without introducing a tone or electric shock. To determine retention of contextual/cued fear memory animals were tested at the acute timepoint, 3 months, and then 6 months after RUS. This involved placing animals in the fear-conditioning spatial context for 3 min and measuring the freezing response using automated video tracking system (Ethovision; Noldus—Netherlands). Freezing responses were calculated by Ethovision Software using the immobility time (2.5 % immobility threshold, 20 frames per second) (Ojo et al. 2014). A cued memory test was conducted 1 h after the contextual fear memory test and involved placing animals in a novel context for 3 min without the tone and a further 3 min with the tone. Freezing response was measured throughout the entire 6 min period.

Open field test

Anxiety and sensorimotor behavior were further assessed by the open field test (Hall and Ballachey 1932). This test was conducted in a circular maze (120cm diameter) setup in a dim lit room (80 Lux). Animals were placed in the center of the maze and the time spent in a predefined center zone and around the walls of the maze was recorded over a 15 min trial.

Elevated plus maze

Anxiety behavior was evaluated using the elevated plus maze (Pellow et al. 1985). The apparatus consists of two open and two closed arms forming a plus shape. The arms are elevated 70cm from the floor. Each mouse was placed on the junction of the four arms of the maze, facing the open arm. The mouse was allowed to freely explore the maze for 5min in a dark light (~1 Lux). The percentage of time spent in the open arms was calculated using Ethovision video tracking system. Animals were retested in a different room at 6 months after RUS.

Forced swim test

The test was performed in a cylindrical container (20 cm height x 20 cm diameters) filled with water at room temperature. Animals were placed in the container for 6 min with video recording from the top. The time spent floating (not swimming nor climbing) was calculated using Ethovision immobility parameter (7.5% immobility threshold, 20 frames per second) as a measure of depression-like behavior. The immobility time measured by

our automated detection settings was comparable to manually calculated floating times by a blinded observer (data not shown).

Radial arm water maze (RAWM)

The RAWM test for spatial learning and memory was conducted as described by Park et al. (2008) (Park et al. 2008). In brief, the test was conducted in a radial arm water maze pool (120cm diameter and 30–40cm height), containing six swim paths extending from an open central arena. A hidden escape platform was placed beneath the end of the target arm and each of the arms was marked with a unique visual cue. Animals were trained for 5 days with 12 trials per day to locate the hidden goal arm. Each trial lasted for 60s. Each entry to a wrong arm was scored as a memory error. The number of errors per block trial, across the entire length of the training session was recorded using a video-tracking system (Ethiovision, Noldus - Netherlands). All animals were subjected to the test at three time points after the traumatic stress (i.e. 2 weeks and 3 months and 6 months after traumatic stress).

Social interaction and novelty recognition tests (Three-chamber test)

The three-chamber test was used to evaluate social behavior. The testing apparatus consists of a rectangular box with three chambers separated by two doors. Each of the outer chambers contains a cage enclosure. The mice were habituated to the testing box for 5 min prior to testing. Testing occurred in two sessions. In the first session, a new intruder mouse (Stranger1) was placed in one cage, while the other cage was left empty. The tested

mouse was placed in the middle chamber and left to explore the three chambers for 10min. The time spent in the Stranger1 chamber versus the empty cage is used to evaluate social interaction of the test subject. In the second session, another intruder mouse (Stranger2) was placed in the second cage along with the previous intruder mouse (Stranger1) still held in the first cage, and the test subject was allowed to freely explore both chambers for 10min. The time spent in the Stranger2 versus Stranger1 chamber was used to evaluate the social memory of the tested animal.

Enzyme-linked immunosorbent assay (ELISA)

To obtain blood specimens, animals were anesthetized with isoflurane, and approximately 500 µl of blood were collected into EDTA tubes by cardiac puncture immediately prior to euthanasia. Samples were centrifuged at 3000g for 3 min, and plasma samples (clear supernatant fraction) were flash frozen in liquid nitrogen and stored at -80 °C. Plasma Corticotropin-releasing hormone (CRH) levels were measured using an enzyme-linked immunosorbent assay (ELISA) purchased from Life-Span biosciences (Seattle, Washington). The Adrenocorticotrophic Hormone (ACTH) ELISA was purchased from Cloud-Clone Corp (Katy, Texas). Tissue and plasma corticosterone levels were measured using an ELISA from Arbor Assays (Ann Arbor, Michigan) and tissue Brain-derived neurotrophic factor (BDNF) levels were measured using an ELISA from Boster Bio (Pleasanton, California). ELISA kits were used as per manufacturers' instructions.

Brain tissue preparation and western blotting

Brains from 6 animals per group were used for biochemical analysis while the brains from the remaining animals were used for hippocampal volume estimation (histopathology). Western blot experiments were conducted as described previously (Ojo et al. 2016). In brief, 6 animals from the stress group and 6 control animals were used for all western blot and ELISA experiments from brain tissue. Tissue was collected following transcardial perfusion by gravity drip with phosphate-buffered saline (PBS). Both hemispheres were extracted and dissected into multiple regions (hippocampus, amygdala, and hypothalamus); these were flash frozen in liquid nitrogen and kept at -80°C .

For western blotting analyses, the hippocampi or amygdalae from both hemispheres were homogenized in 200 μl of M-PER protein extraction reagent containing proteinase and phosphatase inhibitors (ThermoFisher) using a probe sonicator. Samples received 2-s long sonication pulse followed by incubation on ice for 10 s, this process was repeated 3 times. Homogenized samples were spun in a centrifuge at 15000g for 10 minutes (min) and tissue supernatants were collected. Supernatant fractions were denatured at 99°C by boiling in Laemmli buffer (Bio-Rad) containing DDT. Samples were then subsequently resolved on 4% to 15% gradient polyacrylamide criterion gels (Bio-Rad). After electrotransferring, polyvinylidene difluoride membranes were blocked in 5% milk made in Tris-buffered saline and subsequently immunoprobed for different brain-specific primary antibodies overnight (**Table 2.1**). After three washing steps, membranes were probed with horseradish peroxidase-linked secondary antibodies (**Table 2.1**). Anti-GAPDH antibody was used as a housekeeping gene to quantify the amount of proteins electrotransferred, and signal intensity ratios were quantified by chemiluminescence imaging with the ChemiDocTM XRS (Bio-Rad).

Table 2.1. Summary of antibodies (ab) used in western blot experiments

Target	1ry ab dilution	Source	Catalogue #	Host	2ry ab^s dilution
MR	1:1000	Milipore Sigma	MABS496	Mouse	1:2000
GR	1:1000	Cell Signaling Technology	12041	Rabbit	1:2000
FKBP51	1:1000	abcam	ab2901	Rabbit	1:2000
CRH	1:1000	abcam	ab184238	Rabbit	1:2000
TrkB	1:2000	abcam	Ab187041	Rabbit	1:2000
P75NTR	1:1000	Cell Signaling Technology	8238	Rabbit	1:2000
ProBDNF	1:500	Milipore Sigma	MABN110	Mouse	1:2000
GluN1	1:1000	GeneTex	GTX133097	Rabbit	1:2000
GluN2A	1:500	Thermo Fisher Scientific	PA5-27921	Rabbit	1:2000
GluN2B	1:1000	GeneTex	GTX109713	Rabbit	1:2000
PSD95	1:2000	Thermo Fisher Scientific	MA1-046	Mouse	1:2000
CAMKIIα	1:1000	GeneTex	GTX27939	Rabbit	1:2000
NF-κB	1:1000	Cell Signaling Technology	8242	Rabbit	1:2000
pNF-κB	1:500	Cell Signaling Technology	3031	Rabbit	1:2000
GAPDH	1:1000	Cell Signaling Technology	5174S	Rabbit	1:2000

^sAnti-mouse and anti-rabbit secondary antibodies were ordered from Cell Signaling Technology

Multiplex enzyme-linked immunosorbent assay (ELISA)

Levels of 9 cytokines; interferon γ (IFN- γ), interleukin (IL)-1 β , IL-2, IL-4, IL-6, IL-10, IL-12p70, IL-13, and tumor necrosis factor α (TNF α) were measured from a single plasma sample (12.5 μ L) using the MSD proinflammatory Panel I multiplex assay (MesoScale Discovery, Gaithersburg, Maryland).

Neuropeptide magnetic bead panel from hypothalamus

Levels of six neuropeptides (α -MSH, β -Endorphin, Neurotensin, Orexin A, Oxytocin, Substance P) were measured in the hypothalamus using MILLIPLEX MAP Rat/Mouse Neuropeptide Magnetic Bead Panel. The following procedures were implemented to extract neuropeptides from the hypothalamic tissue. Each dissected hypothalamus was dissolved in 200 μ L deionized water and homogenized using a probe sonicator on ice. Immediately after homogenization, 100 μ L of the hypothalamus lysates were mixed with 200 μ L acetonitrile and incubated for 10 min at room temperature then centrifuged at 17,000 g for 5 min. Clear supernatants were collected and dried using speed-vac without sample heating then solubilized in 100 μ L of the manufacture's assay buffer. The rest of multiplex assay steps were carried out as instructed by the manufacturer's guide. Phosphatase and protease inhibitors were added to the remainder of the original lysates and stored for western blot experiments.

Hippocampal volume estimation

5 control animals and 6 stressed animals were used for volume estimation from the hippocampus using Cavalieri principle. At 6 months after last stress exposure, mice were anesthetized with 3% isoflurane and transcardially perfused with a 4% paraformaldehyde phosphate buffered saline (PBS) solution. Brain hemispheres were post-fixed overnight in 4% paraformaldehyde–PBS solution. The volume of the right hippocampus as guided by bregma coordinates (−0.94 to −3.28mm) was determined by quantitative light microscopy using the Cavalieri method as described elsewhere (Ojo et al. 2014). In brief, sagittal sections from the extent of the right hippocampus of each animal (taking every twentieth serial section) were mounted onto superfrost plus slides. An average of 10–15 sections were collected per animal. Mounted sections were air-dried and stained with a solution of 0.1% cresyl fast violet (Tedpella, Reading, CA) and were viewed at low magnification using a DP72 digital camera attached to a motorized Olympus BX63 digital photomicroscope (Olympus, Center Valley, PA). Analyses were carried out blind and were made via the Cell SENS Olympus software package. Digital images were captured electronically and the boundaries of the total dorsal hippocampal compartment and its subfields (DG, CA1, and CA2/CA3) were digitally outlined on each section from the series of sagittal sections of the right hemisphere. For each animal, the total volume of the right hippocampus and its subfields were subsequently derived by multiplying the calculated total surface area by the mean distance between the series of sections. Data are expressed as mean volume (in mm³) ± SEM.

Statistical Analysis

The relationships between stress and control animals for Western blotting, ELISA, and neurobehavioral data were assessed using a t-test predefined criterion of $p < 0.05$ to assess group differences, while experiments examining stress effects over time were assessed using a RM-ANOVA. Pearson correlation and Spearman correlation were used for parametric and nonparametric data sets, respectively. The choice of the appropriate statistical test was made after assessment of normal distribution using the Shapiro-Wilk normality test. All analyses were performed with Graph Pad prism version 7.0a statistical software (La Jolla, California). Statistical outliers were identified using Grubbs' test and excluded.

2. 3 Results

Mice exposed to the 21-day stress paradigm show attenuation of bodyweight gain and anxiety-like behavior immediately after stress.

Stress is usually associated with changes in bodyweight and food intake (Harris 2015). Therefore, I obtained the weekly bodyweight for each mouse to examine the effects of the RUS paradigm (**Figure 2.3A**). Stressed mice showed reduced weight gain in the first 3 weeks of stress (RM-ANOVA: interaction $F_{(4, 116)} = 12.54$, $p < 0.0001$, with the Sidak post hoc test) compared to their unexposed group. The changes in bodyweight are mainly driven by an intense decrease in their growth rate during the first week of stress (RM-ANOVA: interaction $F_{(3, 87)} = 10.58$, $p < 0.0001$, with the Sidak post hoc test, **Figure 2.3B**).

In addition, stressed mice showed an anxiety-like behavior as evident by reduced center zone entries per 3 min time bins (RM-ANOVA: Stress effect $F_{(1, 18)} = 27.87$, $p < 0.0001$, with the Sidak post hoc test) and throughout the entire 15 min (t-test: $t_{(18)} = 5.174$, $p < 0.0001$) in the open field test (**Figure 2.3C and 2.3D**) at the acute timepoint. However, assessment in the elevated plus maze (EPM) in the same timepoint showed no significant differences between groups in the open arm duration (t-test: $t_{(18)} = 1.8$, $p = 0.083$, **Figure 2.3E**) or open arm entries (t-test: $t_{(18)} = 1.8$, $p = 0.99$, **Figure 2.3F**).

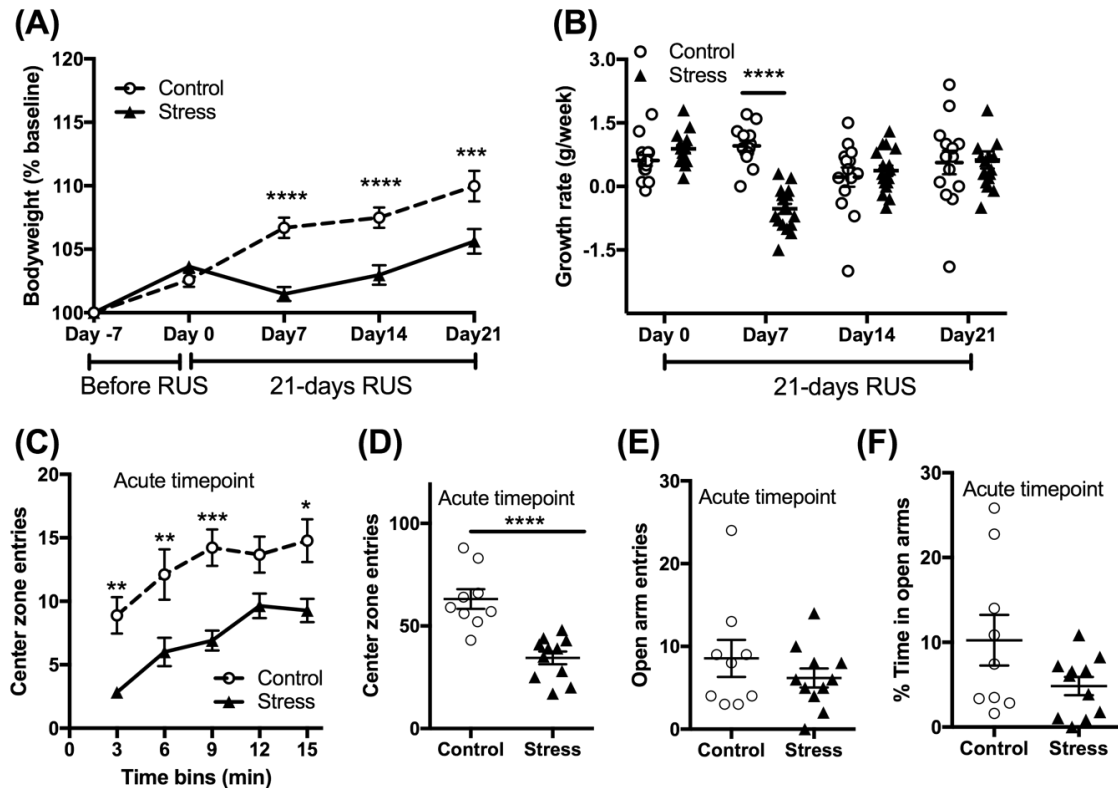


Figure 2.3 Effect of RUS on bodyweight gain and anxiety-like behavior. During the 21-day stress paradigm, stressed mice showed a reduction in body weight gain when compared to control mice (A). Growth rate was significantly lower only during and after the first week of stress (day7) (B). Exposure to stress increased anxiety-like behavior at the acute timepoint as evident by reduced center zone entries per a 3- min time bin (C) and in the total 15 min (D) in the open field test. No significant changes were observed in the EPM open arm time (E) and entries (F) at the acute timepoint. Data in (A, B) were analyzed using repeated measures Two-Way ANOVA followed by post-hoc Tukey's test ($n = 14-17$), while data in (C-F) were analyzed using a student t-test ($n=8-9$). Asterisks denote statistical significance as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Stressed mice recall fear memory and show passive stress coping behavior at 6 months after RUS.

Fear conditioning using electric footshocks was used as a stressor in the RUS paradigm and also as a tool to evaluate the recall of contextual and cued fear memory responses (Ojo et al. 2014). Stressed mice showed a significant increase in their freezing (Immobility time) when placed in the same trauma context at the acute timepoint (t-test:

$t_{(21)}=8.4$, $p<0.0001$, **Figure 2.4A**). However, stressed mice were not able to recall contextual fear memory when examined at 3 months and 6 months after RUS (**Figure 2.4B and 2.4C**, $p>0.05$). I also examined recall of cued fear memory at the same timepoints. Mice were placed in a novel context for 3 minutes, and then exposed to a trauma related cue (auditory tone) lasting an additional 3 minutes. While all animal groups showed minimal freezing during the first 3 min in the novel context (without a tone), stressed animals showed an increased % freezing upon tone introduction at the acute timepoint (Two-way ANOVA, interaction $F_{(1, 40)}=111.8$, $p<0.0001$, **Figure 2.4D**), 3 months (Two-way ANOVA, interaction $F_{(1, 40)}=8.7$, $p<0.0052$, **Figure 2.4E**) and 6 months (Two-way ANOVA, interaction $F_{(1, 40)}=5.4$, $p<0.025$, **Figure 2.4F**) after RUS. Chronic changes in stress-coping strategies and anxiety-like behavior were also examined at 6 months after RUS using the forced swim test and the elevated plus maze, respectively. As depicted in **Figure 2.4G and 4H**, stressed animals showed negative stress coping behavior as evident by increased immobility time in the forced swim test (t-test: $t_{(20)}=2.5$, $p=0.02$, **Figure 2.4H**). No significant differences were observed between control and stressed mice at 3 months and 6 months after RUS in the number of open arm entries in the elevated plus maze ($p>0.05$, **Figure 2.5A and Figure 2.5I**) or center zone entries in the open field test ($p>0.05$, **Figure 2.5B**). It is important to note that the performance of mice in the EPM test and the suitability of the EPM and Open field for retesting is questionable (Hussin et al. 2012; Walf and Frye 2007). Control mice in the EPM always performed poorly (spending only few seconds in the open arms) which reduced the window for detecting a significant effect between sham and stressed mice. Additionally, although some studies suggested changing the retesting room and separating the trials by four weeks to improve retesting reliability (Schneider et al. 2011), our data indicated that control mice show fewer entries

to the open arms of EPM and center zone of the Open Field upon retesting at different rooms 3 months and 6 months after RUS. (Figure 2.5A, and 2.5B).

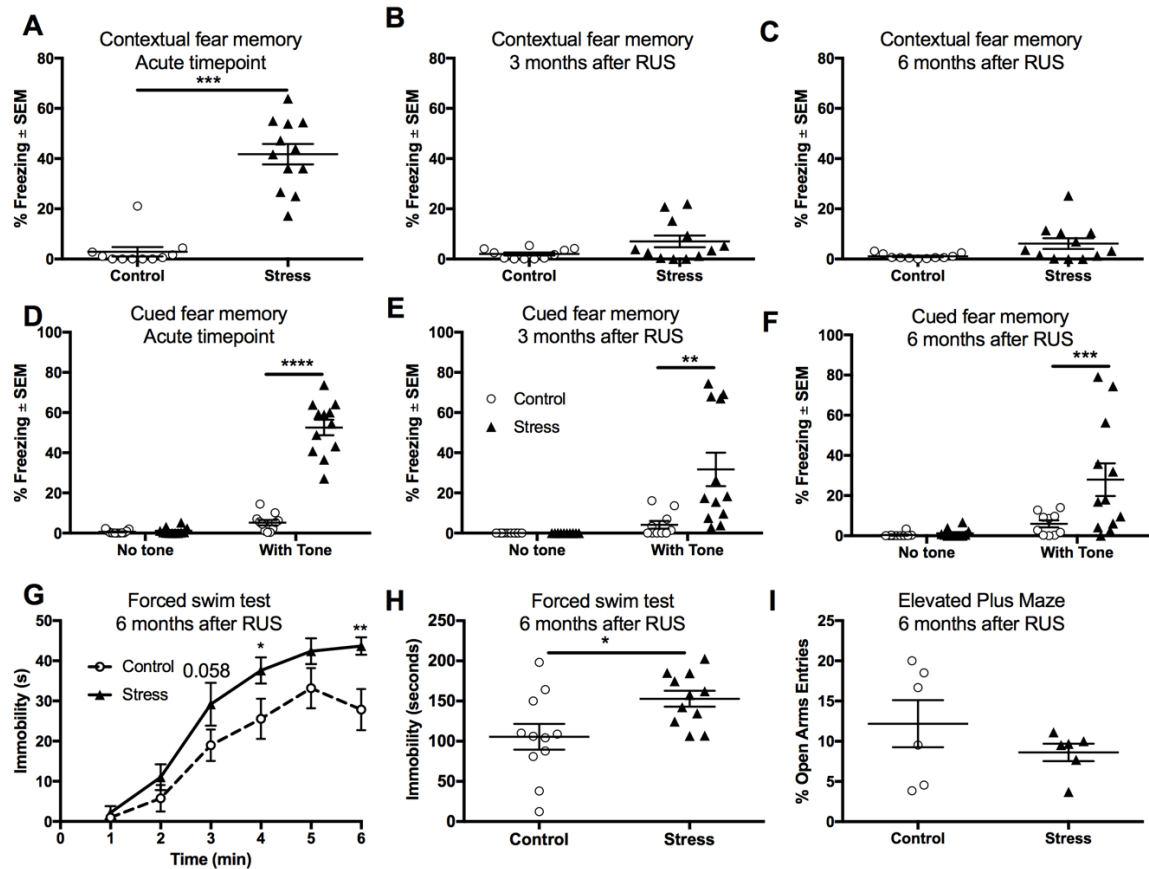


Figure 2.4. Recall of contextual and cued fear memory at the acute timepoint, 3 and 6 months after RUS. Stressed animals recalled contextual fear memories only at the acute timepoint as evident by increased freezing time compared to controls at the acute timepoint (A), but not at 3 months (B) or 6 months (C) after RUS. Panels E-G depict % freezing scores in the first minute of the cued memory test in absence and presence of a cue (tone) at the acute, 3-month and 6-month timepoints, respectively. There was no significant freezing for all treatment groups when tested in a new context without cues (no tone) at all timepoints (D-F). After tone introduction, RUS mice showed a significant increase in % freezing at the acute timepoint, 3 months and 6 months after RUS (D-F). Stressed mice displayed increased immobility time in min 4 and 6 of the FST when compared to a control group (G). Average immobility time in the last 4 min was increased in stressed mice compared to the control group (H). RUS didn't alter open arm entries in the elevated plus maze test at 6 months after stress. Data in (A-C, H, I) were analyzed using a student t-test (n=6-12), while data in D-F were analyzed using repeated measures Two-Way ANOVA followed by post-hoc Sidak test (n=10-12). Asterisks denote statistical significance as follows: *p < 0.05; **p < 0.01; ***p < 0.001.

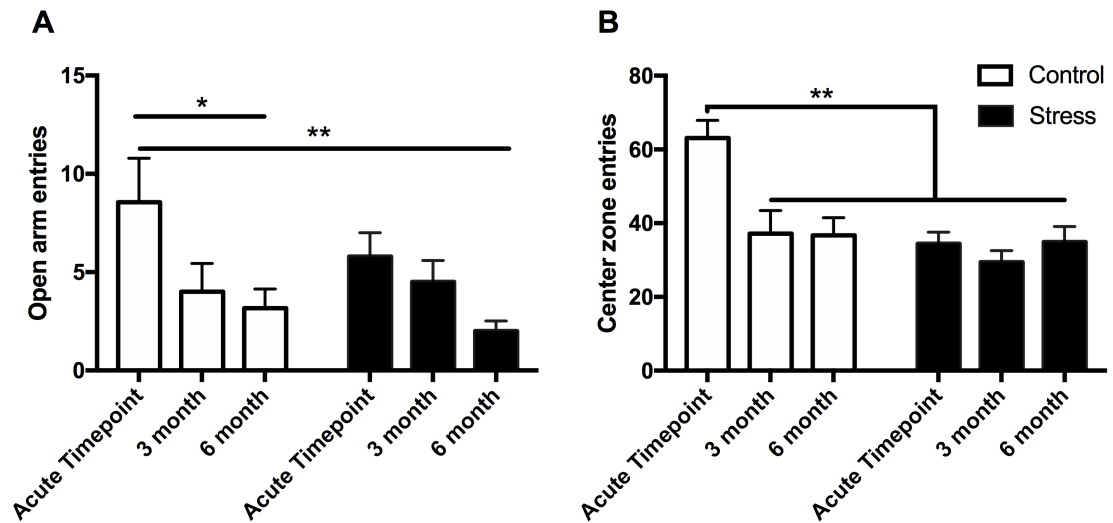


Figure 2.5. Effect of repeated testing on mice performance in the EPM and Open Field tests. (A) Control mice made fewer entries to the open arm of the EPM upon retesting at 6 months after RUS. In the Open field test (B), control mice made fewer entries to the center zone when retested at 3 months and 6 months after RUS. No significant differences were observed between control and stressed animals in both tests at the 3 month and the 6 month timepoints. Data were analyzed using Two-Way ANOVA.

Contextual memory retrieval is thought to be a hippocampal-dependent task (Maren and Holt 2000). Since stressed mice were able recall cued fear memory, but not contextual fear memory at 3 months and 6 months after RUS, I examined the effect of stress on other hippocampal-related tasks such as social memory and spatial learning. Mice exposed to RUS did not show any impairment in social memory at the acute timepoint, 3 months or at 6 months after RUS when tested using social novelty recognition tests ($p > 0.05$, **Figure 2.6**). Our findings in the social memory test at the chronic timepoints are inconclusive because control mice did not show any preference to the novel mouse, (Stranger2), possibly due to retesting (**Figure 2.6B**). There were however no impairments in spatial learning and memory in stressed animals at any timepoint when tested using the radial arm water maze ($p > 0.05$, **Figure 2.7**).

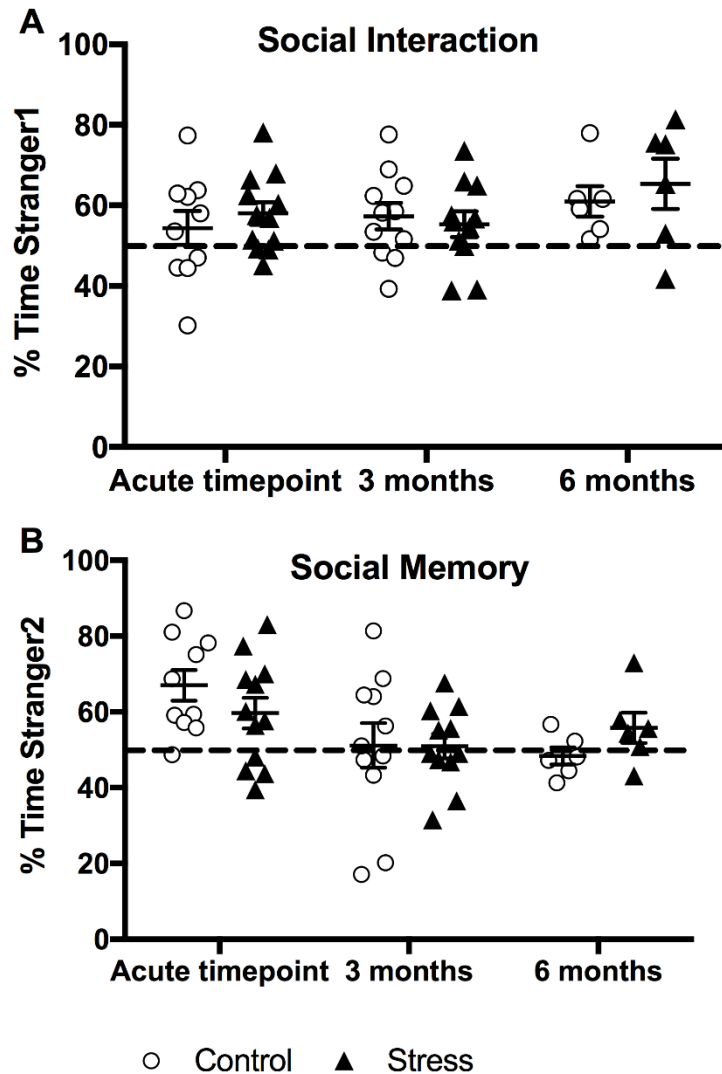


Figure 2.6. Effect of stress on social interactions and social memory at the acute timepoint, 3 months and 6 months after RUS. No differences in social interactions (A) or social memory (B) were observed between RUS animals and controls at the acute timepoint, 3 months or 6 months after RUS. In (A), the ratio between time spent around stranger1 cage to total time spent around stranger1 and the empty cage was calculated for each animal to evaluate social interactions, while in (B), the ratio between time spent around stranger2 cage (new never seen mouse) to the total time around both strangers was calculated to evaluate social memory (social novelty recognition).

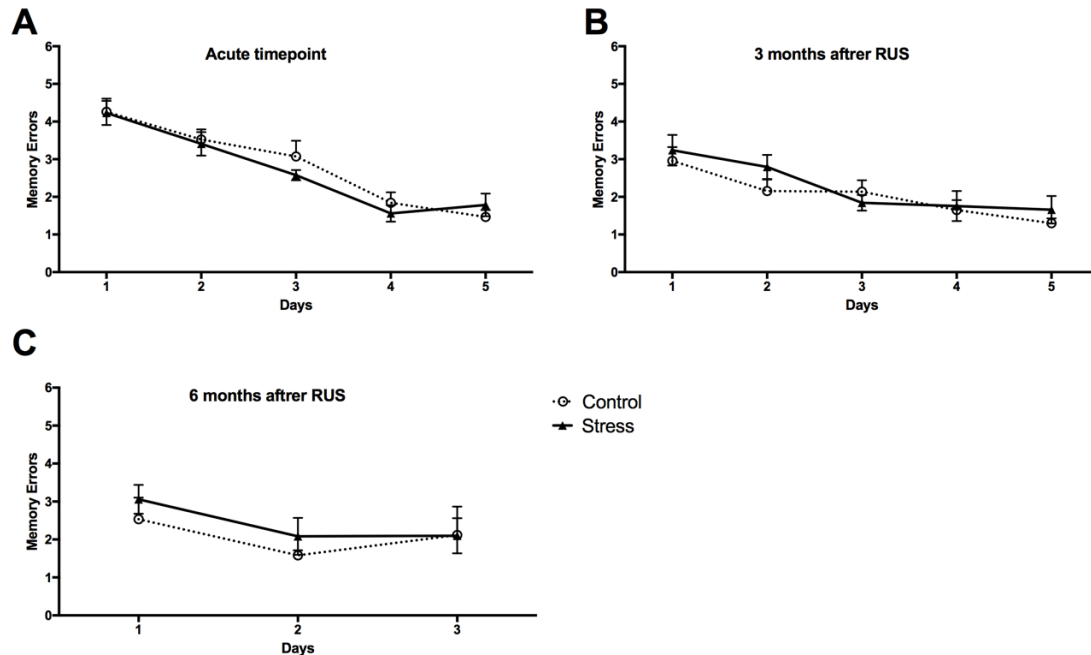


Figure 2.7. Effect of stress on spatial learning and memory at the acute timepoint, 3 months and 6 months after last stress. Panels A, B and C depict the performance of each group during three separate training sessions in a radial arm water maze (RAWM) at the acute timepoint, 3 months and 6 months after last stress. All the animal groups demonstrated a learning curve at all timepoints after trauma. No significant differences between the tested groups were observed at any timepoint. Data were analyzed using repeated measures Two-Way ANOVA. Tukey's multiple comparisons post-hoc test was performed in all cases.

Stressed mice display lower plasma corticosterone levels 6 months after RUS; suggesting blunted HPA-axis reactivity.

In order to examine the effect of stress on the HPA axis, baseline plasma corticosterone levels were measured at the end of the light cycle 6 months after RUS. I found that mice exposed to stress showed significantly lower plasma corticosterone levels (t-test: $t_{(21)} = 2.9$, $p = 0.009$, **Figure 2.8A**) compared to control mice. Similarly, ACTH levels were significantly lower in stressed mice compared to controls (t-test: $t_{(14)} = 2.3$, $p = 0.04$, **Figure 2.8B**). No significant change was seen in plasma CRH at 6 months ($p > 0.05$, **Figure**

2.8C). I subsequently examined glucocorticoid signaling in the hypothalamus by measuring the expression levels of glucocorticoid receptors and regulatory proteins (**Figure 2.8D-H**). While there was no change in expression levels of glucocorticoid receptor (GR), mineralocorticoid receptor (MR) or CRH levels, the levels of FK506 binding protein 51 (FKBP51) were downregulated in the hypothalamus of stressed mice relative to controls (t-test: $t_{(9)}=2.4$, $p=0.042$, **Figure 2.8D and 2.8G**). To explore the relationship between abnormalities in the HPA axis function and behavioral outcomes in our model, I assessed the statistical correlation between individual mouse corticosterone plasma levels and their corresponding neurobehavioral scores in both control and stress groups. I found a significant negative correlation between plasma corticosterone levels and freezing scores in the cued fear memory test (Spearman correlation: $r=-0.749$, $p=0.001$, **Figure 2.8I**) and the immobility time in the forced swim test (Pearson correlation: $r=-0.63$, $p=0.0022$, **Figure 2.8J**). Furthermore, cued fear memory freezing scores correlated positively with the immobility time in the forced swim test (Pearson correlation: $r=-0.479$, $p=0.0278$, **Figure 2.8H**).

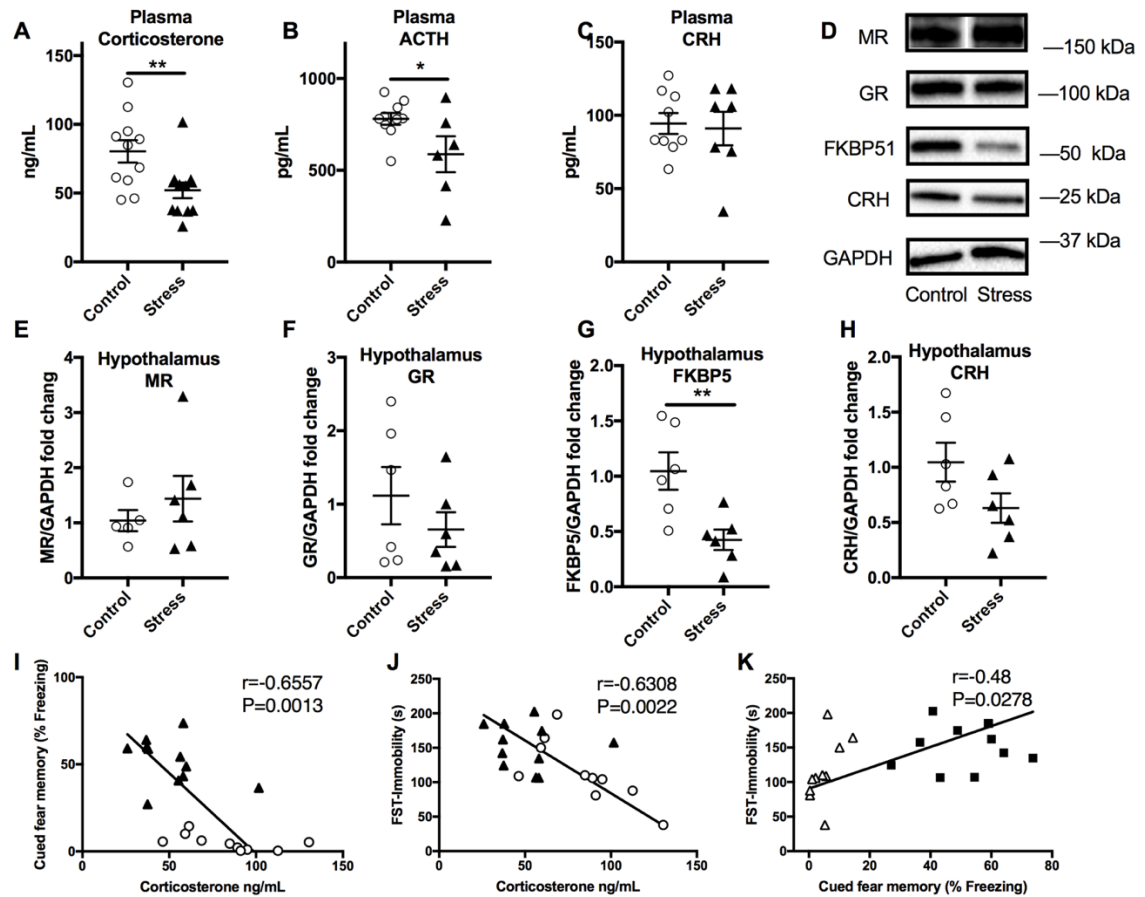


Figure 2.8. Effect of stress on HPA axis regulation at 6 months after RUS. Mice exposed to stress showed lower plasma levels of corticosterone (A) and ACTH (B), but not CRH (C) when compared to a control group at 6 months after RUS (n=8-12). Representative western blot images for markers of glucocorticoid signaling in the hypothalamus (D). Quantification of mineralocorticoid Receptor (E), glucocorticoid receptor (F), FKBP5 (G) and CRH (H) levels in the hypothalamus (n=5-6). Only the levels of FKBP5 (G) were significantly reduced after RUS. Plasma corticosterone levels were inversely correlated with the % freezing scores in the cued fear memory test (I) and the immobility time in the forced swim test (J) (n=21). There was a positive correlation between % freezing scores in the cued fear memory test and the immobility time in the forced swim test (K). Each solid black triangle represents an animal in the stress group, while white circles represent control animals. Asterisks denote statistical significance as follows: * $p < 0.05$; ** $p < 0.01$.

Previous studies have suggested interplay between the endocrine and inflammatory/immune systems in the pathophysiology of depression (Griffin, Charron, and

Al-Daccak 2014) and PTSD (Griffin et al. 2014), owing partly to the immunoregulatory role of corticosterone. To examine the effect of our RUS paradigm on the peripheral immune system, I evaluated the levels of plasma cytokines at 6 months after stress. I found a 2-fold increase in plasma levels of pro-inflammatory IL1 β (t-test: $t_{(8)}=2.4$, $p=0.04$, **Table 2.2**) and IFN γ (t-test: $t_{(7)}=3.9$, $p=0.006$, **Table 2.2**), while the levels of other cytokines were not significantly altered (**Table 2.2**).

Table 2.2. Effect of stress on plasma cytokines at 6 months after RUS

Cytokine	Fold Change ^s \pm SD	p-value	n	Time after stress
IFN γ *	1.97 \pm 0.26	0.04*	5	6 months
TNF α	1.02 \pm 0.82	0.88	5	6 months
IL1 β **	1.94 \pm 0.07	0.006**	5	6 months
IL2	1.15 \pm 0.27	0.688	5	6 months
IL5	0.84 \pm 0.57	0.613	5	6 months
IL6	1.25 \pm 1.37	0.431	5	6 months
IL10	0.83 \pm 1.30	0.132	5	6 months
IL12	1.65 \pm 4.96	0.276	5	6 months

^sPlasma cytokines levels in stressed animals relative to controls \pm standard deviation

As the hypothalamus secretes numerous neuropeptides that regulate several autonomic functions implicated in regulating neurocircuitry pathways involved in stress management, I measured the expression levels of various neuropeptides in the hypothalamus using a magnetic bead multiplex assay. As shown in **Figure 2.9**, there was no significant difference in the expression levels of Oxytocin, β -endorphin, Neurotensin, α -MSH, and Substance P (**Figure 2.9**, $P>0.05$). Interestingly, Orexin-A levels were significantly elevated in stressed mice compared to controls (t-test: $t_{(10)}=2.437$, $p=0.035$, **Figure 2.9A**).

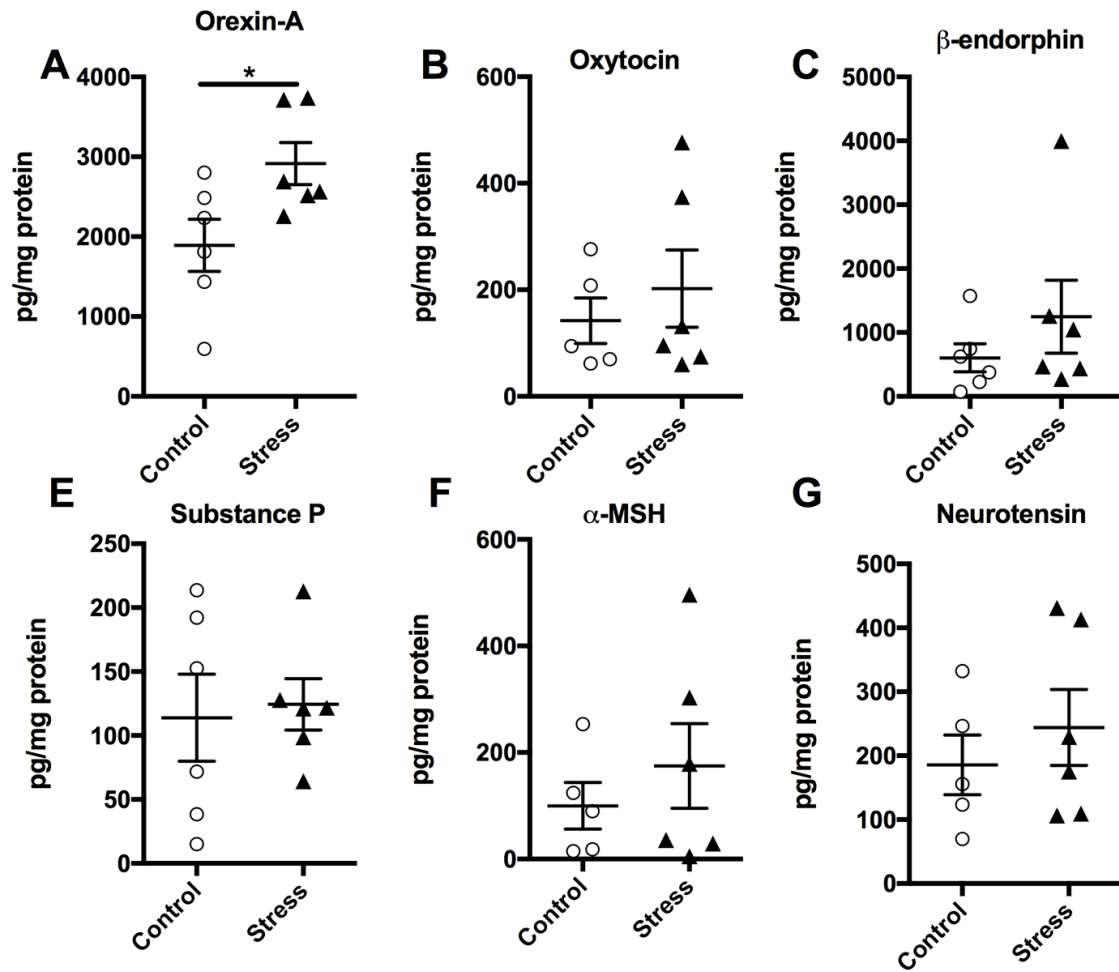


Figure 2.9. Exposure to stress elevates Orexin-A levels in the hypothalamus. Magnetic beads multiplex assay showed an increase in the hypothalamic levels of Orexin-A at 6 months after RUS. No significant changes were observed in the levels of Oxytocin (B), β -endorphin (C), Substance P (E), α -MSH (F), and Neurotensin (G). Data in Figure 2.9 were analyzed using a student t-test ($n=5-6$). Asterisks denote statistical significance as follows: $*p < 0.05$.

Effect of stress on the hippocampus at 6 months after RUS

The hippocampus is prone to negative consequences of stress (McEwen, Nasca, and Gray 2016) and it is postulated that corticosteroids are primary mediators of these stress effects (Roth et al. 2012). Consistent with our plasma findings, corticosterone levels

were also reduced in the hippocampal tissue lysates of stressed mice compared to controls (t-test: $t_{(10)}=2.34$, $p=0.044$, **Figure 2.10A**). To further examine the chronic effect of RUS on the hippocampus, I estimated the volume of the hippocampus and its subfields and measured the levels of a number of synaptic plasticity markers. Using the Cavalieri stereology principle for volume estimation, I found that the volume of the CA1 region of the dorsal hippocampus (t-test: $t_{(9)}=2.34$, $p=0.044$, **Figure 2.10B**) was significantly reduced in stressed animals compared to controls at 6 months after stress. In addition, there was a trend towards reduced total dorsal hippocampus volume (t-test: $t_{(9)}=2.13$, $p=0.061$, **Figure 2.10C**) in stressed animals, and no significant change in CA2 and CA3 or Dentate gyrus regions ($p>0.05$, **Figure 2.10B**). I then examined the expression levels of notable markers/mediators of neuroplasticity using immunoblot analysis of hippocampal lysates. BDNF levels were downregulated in mice exposed to RUS (t-test: $t_{(9)}=3.55$, $p=0.006$, **Figure 2.10D**), while the levels of other markers such as PSD95, CAMKII, NGF, TRKB, ProBDNF and NMDA receptors were not significantly altered after RUS ($P>0.05$, **Figure 2.11**). Interestingly, CRH levels were elevated in animals exposed to stress (t-test: $t_{(10)}=2.4$, $p=0.037$, **Figure 2.10I**) at 6 months after RUS. Moreover, I did not observe any significant change in glucocorticoid signaling receptors (GR and MR) or related binding proteins (FKBP51) at 6 months (**Figure 2.10E-I**).

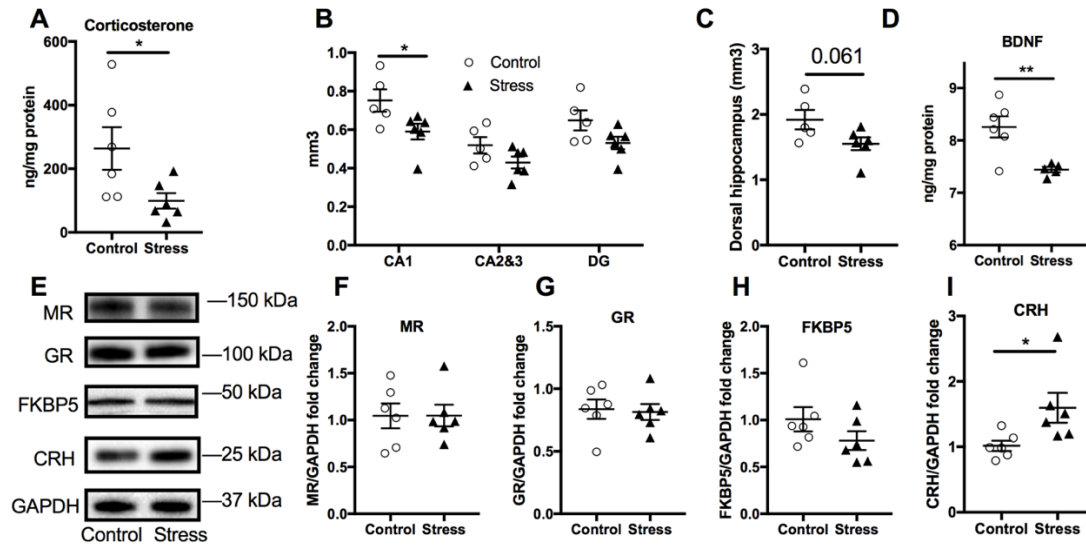


Figure 2.10. Effect of stress on the hippocampal volume and HPA axis markers at 6 months after RUS. Effect of stress on the volume of the dorsal hippocampus (A) and the volume other hippocampal regions (B). Stress resulted in a volume reduction only in the CA1 region (B). Hippocampal BDNF (C) and corticosterone (D) levels were also reduced at 6 months after stress. Representative western blot images for markers of glucocorticoid signaling in the Hippocampus (E). Quantification of mineralocorticoid receptor (F), glucocorticoid Receptor (G), FKBP5 (H) and CRH (I) levels in the hippocampus at 6 months after RUS. Only the levels of CRH (I) were significantly increased after RUS. Data were analyzed using a student t-test (n=5-6). Asterisks denote statistical significance as follows: *p < 0.05; **p < 0.01.

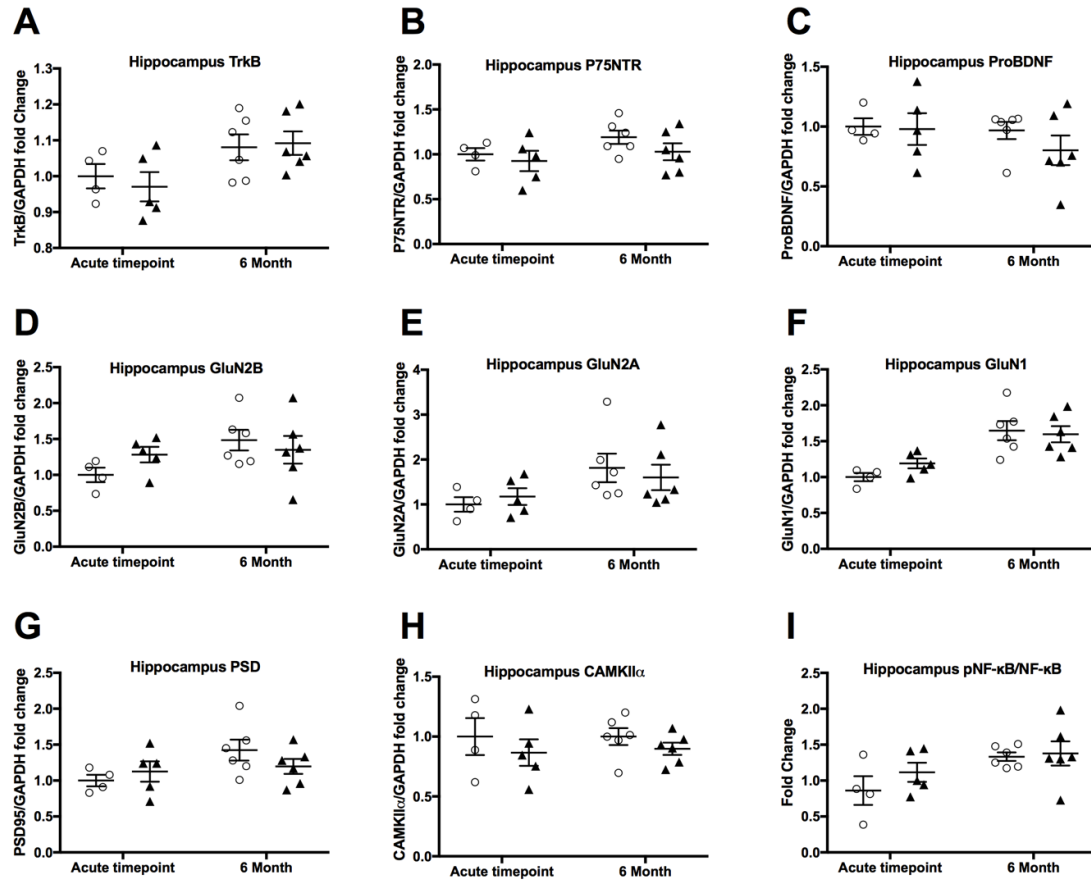


Figure 2.11. Effect of stress on synaptic plasticity markers in the hippocampus at the acute time point and at 6 months after RUS. Quantification of western blot images of TRKB (A), P75NTR (B), ProBDNF (C), GluN2B (D), GluN2A (E), GluN1 (F), PSD95 (G), CAMKII α (H), and pNF- κ B/NF- κ B (I), levels in the hippocampus at the acute timepoint and at 6 months after RUS. All Data were analyzed using a student t-test (n=4-6).

Effect of stress on markers of neuroplasticity in the amygdala

The amygdala is associated with emotional learning and cued fear memory and is also prone to negative consequences of stress (McEwen et al. 2016). Therefore, I probed for the levels of a number of markers associated with neuroplasticity using immunoblotting. No significant changes were observed in amygdala lysates at 6 months after stress ($p >$

0.05, **Figure 2.12**). However, I observed that lysates from the acute timepoint showed significant changes in several markers associated with neuroplasticity.

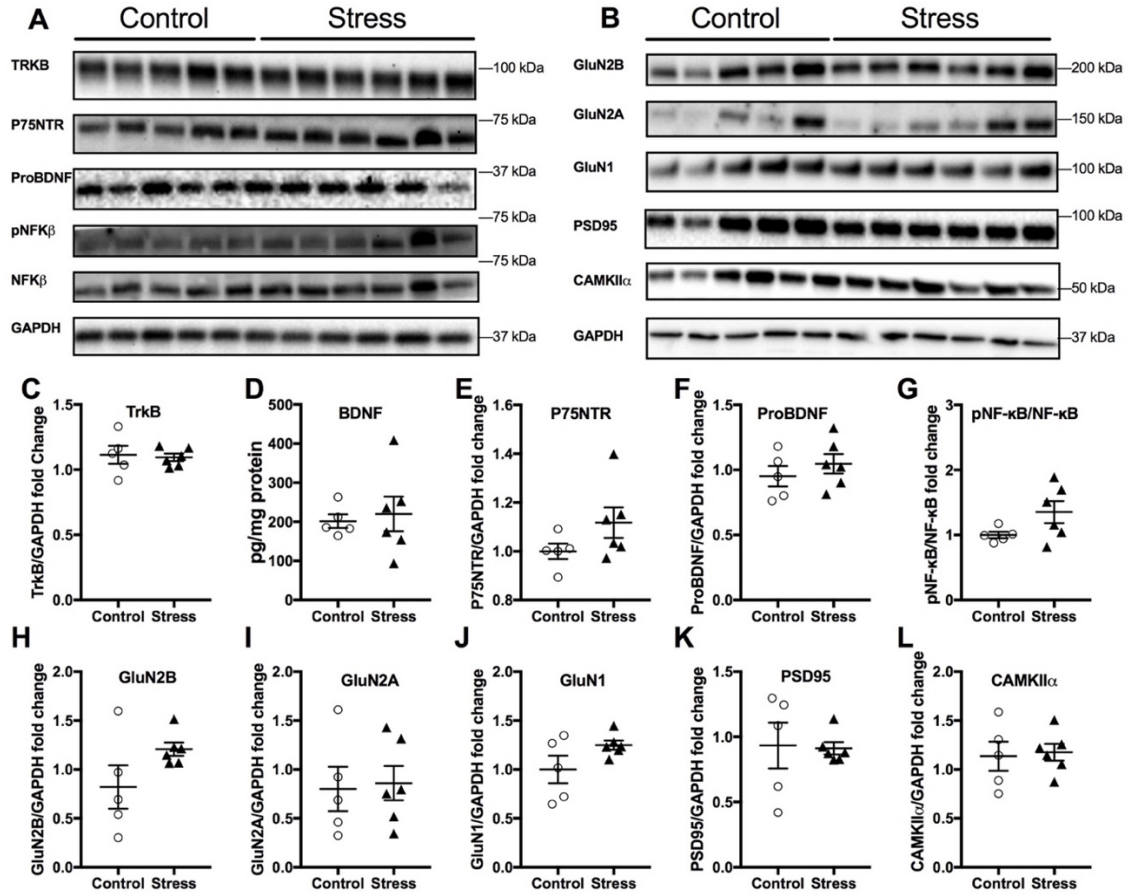


Figure 2.12. Effect of stress on synaptic plasticity markers in the amygdala at 6 months after RUS. Representative western blot images from amygdala lysates for neurotrophic markers, NMDA receptors and other synaptic plasticity markers are shown in panels (A), and (B). Quantification of western blot images of TRKB (C), P75NTR (F), ProBDNF (F), pNF-κB/NF-κB (G), GluN2B (H), GluN2A (I), GluN1 (J), PSD95 (K), and CAMKII (L) levels in the amygdala at 6 months after RUS. All Data were analyzed using a student t-test (n=5-6).

As shown in **Figure 2.13**, there was a significant reduction in the levels of ProBDNF (t-test: $t_{(7)} = 2.64$, $p = 0.034$, **Figure 2.13E**) and its signaling receptor, P75NTR (t-test: $t_{(7)} = 4.02$, $p = 0.0052$, **Figure 2.13D**). In addition, levels of NMDA receptor type1

(GluN1) were reduced (t-test: $t_{(7)} = 6.15$, $p = 0.0003$, **Figure 2.13I**), including downstream signaling kinase, CAMKII (t-test: $t_{(7)} = 4.5$, $p = 0.003$, **Figure 2.13L**) and spine density protein marker, Post Synaptic Density protein 95 (PSD95) (t-test: $t_{(7)} = 3.17$, $p = 0.016$, **Figure 2.13J**) in animals exposed to stress compared to controls. No significant changes were observed in the amygdalar levels of BDNF or its receptor, Trkb ($p > 0.05$, **Figure 2.13B and 2.13C**); neither were there any changes in other NMDA receptor subunits such GluN2B and GluN2A ($p > 0.05$, **Figure 2.13G and 2.13H**) or in the ratio of phosphorylated nuclear factor κ B to nuclear factor κ B (pNF- κ B/NF- κ B).

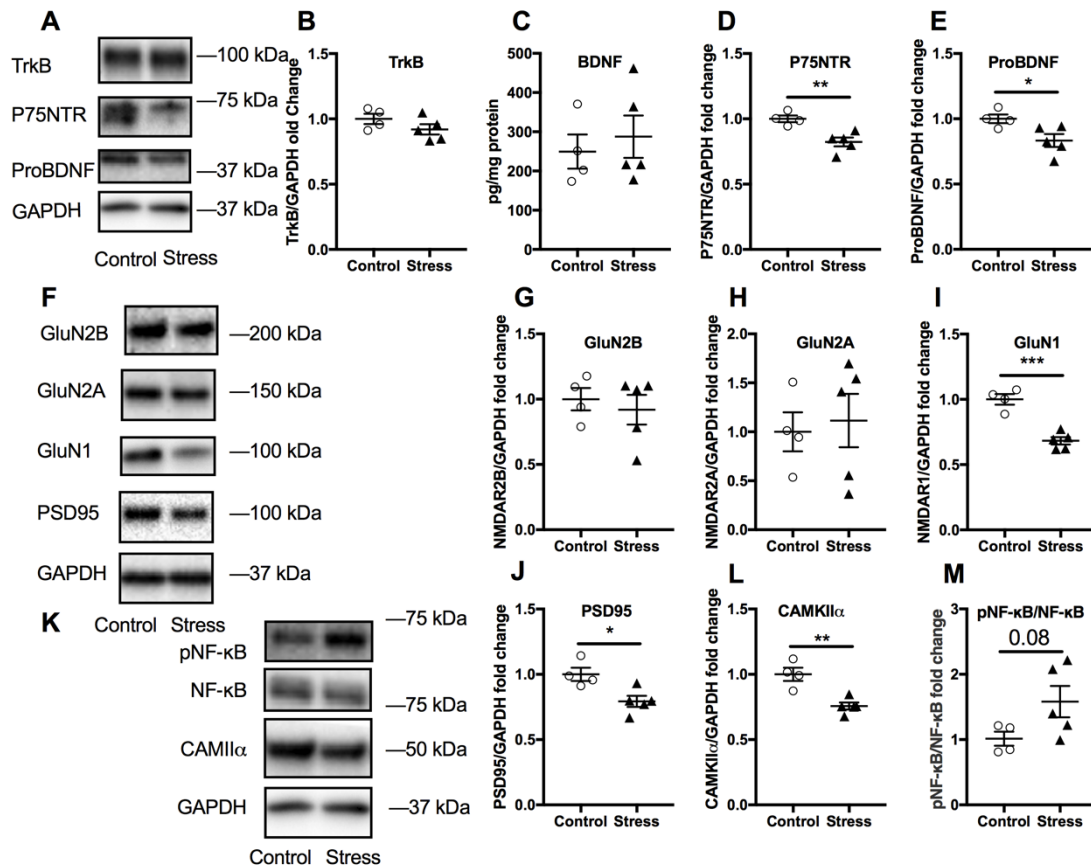


Figure 2.13. Effect of stress on synaptic plasticity markers in the amygdala at the acute timepoint. Representative western blot images from amygdala lysates for neurotrophic markers, NMDA receptor subunits and other synaptic plasticity markers are shown in panels (A), (F) and (K), respectively. Quantification of western blot images of TRKB (B), P75NTR (D), ProBDNF (E) and GluN2B (G), GluN2A (H), GluN1 (I), PSD95

(J), CAMKII (L), and pNF- κ B/NF- κ B (M) levels in the amygdala at the acute timepoint. All Data were analyzed using a student t-test (n=5-6). Asterisks denote statistical significance as follows: *p < 0.05; **p < 0.01.

Effect of stress on the hippocampus and the hypothalamus at the acute timepoint.

No significant changes were observed in GR, FKBP, CRH, and BDNF in the hypothalamus and the hippocampus at the acute timepoint (p>0.05, **Figure 2.14**).

There was a trend towards decreased hippocampal BDNF levels and increased hypothalamic FKBP5 levels in stressed animals at the acute timepoint. However, these findings were not statistically significant. A summary of the findings presented in this study is depicted in **Figure 2.15**.

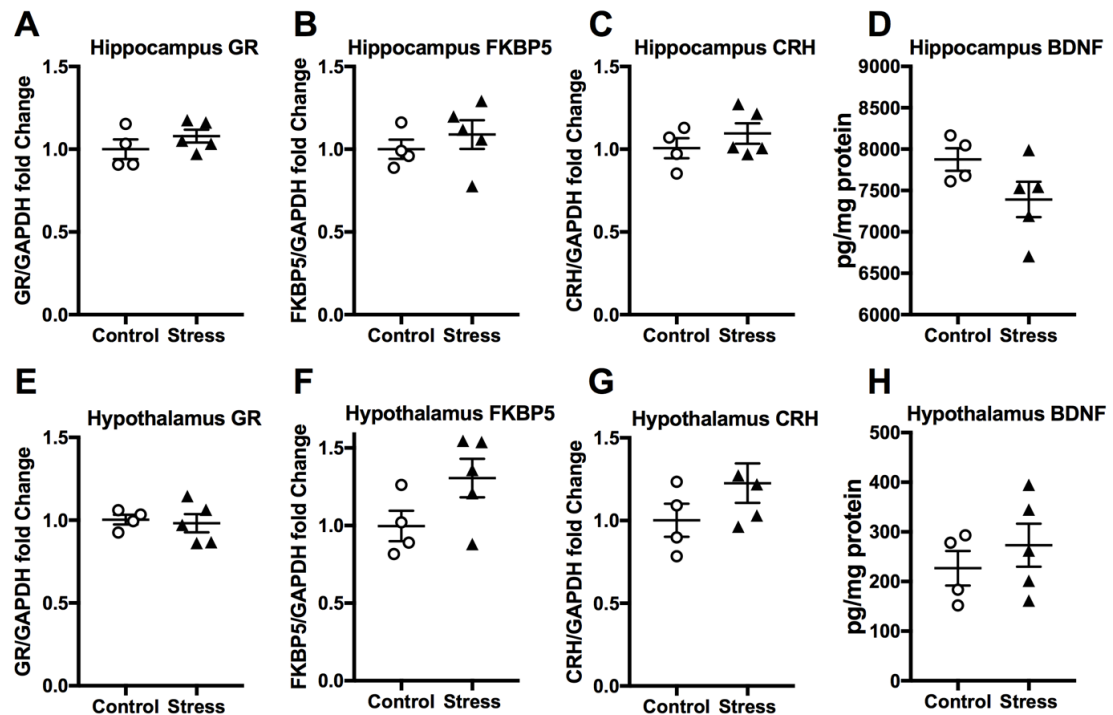


Figure 2.14. Effect of stress on the hippocampal and the hypothalamic HPA axis markers at the acute timepoint. Quantification GR (A), FKBP5 (B) and CRH (C), and (D) BDNF levels in the hippocampus at the acute timepoint. The lower panels depict the

quantification of different markers from the hypothalamus at the same timepoint; GR (E), FKBP5 (F), CRH (G), and (H). BDNF levels was measured using a commercial ELISA, while the rest of the markers were measured using western blotting. Data were analyzed using a student t-test (n=5-6). Asterisks denote statistical significance as follows: *p < 0.05; **p < 0.01.

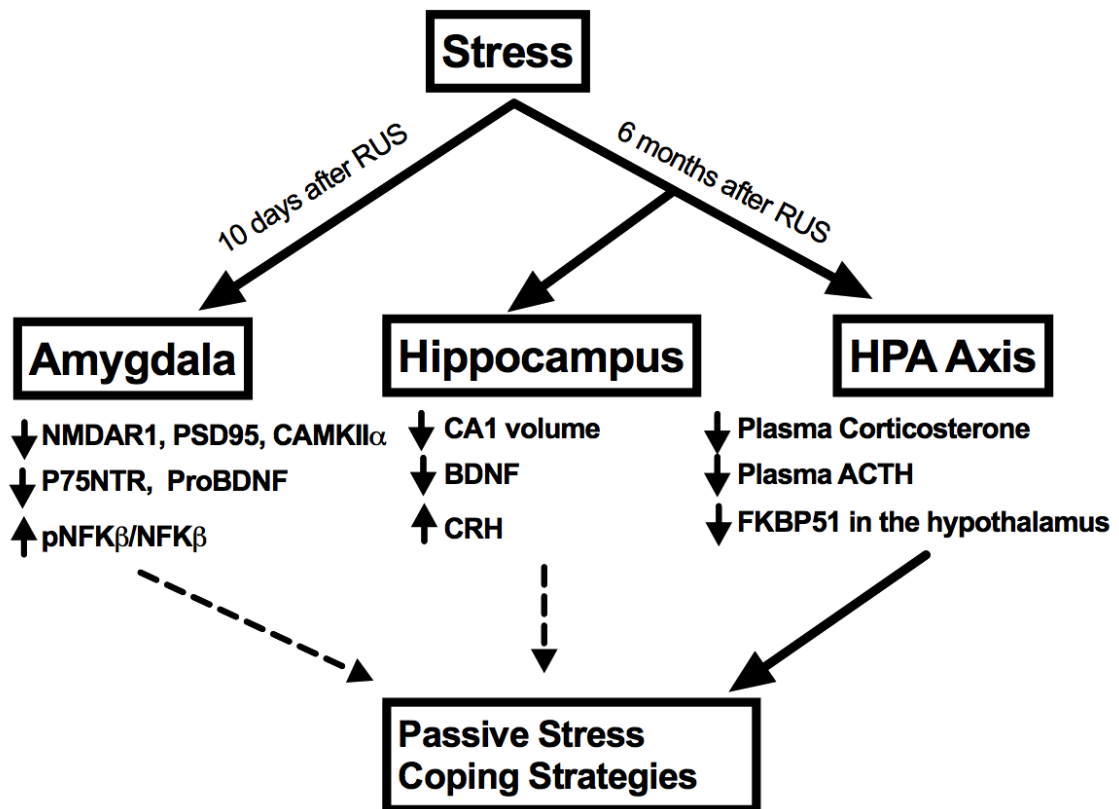


Figure 2.15. A summary of stress effects on the amygdala, hippocampus and HPA axis at different timepoints after RUS.

2. 4 Discussion

In our new RUS paradigm, I assessed the chronic neurobehavioral, neuroendocrine and neurobiological consequences of a repeated unpredictable stress (RUS) paradigm in mice. The purpose of our study was to develop a mouse model of stress with relevance to PTSD that demonstrates chronic behavioral deficits (face validity) with validated pathophysiological measures (construct validity). Using a battery of behavioral tests, I showed that animals exposed to RUS demonstrate traits similar to the human condition; particularly acute anxiety (**Figure 2.3C-D**), recall of traumatic memory cues (**Figure 2.4D-F**) and negative alterations in stress coping strategies (**Figure 2.4G-H**) at 6 months after RUS.

Blunted HPA axis responsiveness (Yehuda 2001; Yehuda et al. 1993) (measured as lower cortisol levels compared to control subjects) and reduced hippocampal volume (Bremner et al. 1995; Levy-Gigi et al. 2013) have been frequently reported in PTSD human subjects. Our findings of lower plasma corticosterone levels and reduced hippocampal CA1 volume 6 months after RUS (**Figures 2.8A** and **2.10B**) support the construct validity of our model.

Because I have utilized a prolonged unpredictable repeated stress paradigm in this study, a caveat of this work is the ability to distinguish between PTSD and other related neuropsychiatric conditions such as depression. Although there is a considerable symptom overlap between PTSD and depression the nature of HPA axis dysregulation is quite different (Schöner et al. 2017). Increased plasma cortisol levels is a common finding in human patients with melancholic depression (a form of major depressive disorder), (Gold, Machado-Vieira, and Pavlatou 2015; Palazidou 2012). Interestingly, normal or decreased

HPA axis function is found in atypical depression patients suggesting that the HPA axis activity is dependent on depression subtypes (Juruena et al. 2018). In animal studies, exposure to chronic mild to moderate stress is commonly used to study depression (MCEWEN 2004; Strekalova et al. 2004). For example, chronic unpredictable stress (CUS) paradigms involve random, unpredictable exposure to variable daily stressors such as restraint, cage tilting and shaking for 4 to 8 weeks. CUS models typically show anxiety, depression-like behavior and increased plasma corticosterone levels at one, 6 and 18 days after stress cessation (Abd El Wahab, Ali, and Ayuob 2018; Monteiro et al. 2015). Interestingly, C57BL/6 mice appear to be more resilient to CUS compared to other mouse strains and longer stress durations are usually needed to show depression-like behavior (Monteiro et al. 2015). Blunted HPA axis reactivity to acute stressors has been reported in repeated immobilization (Harvey et al. 2006; Liberzon et al. 1997), social defeat (Beitia et al. 2005) and predator exposure models (Zoladz et al. 2015). In addition, social isolation has been linked to reduced plasma baseline corticosterone levels in mice (Arndt et al. 2009; Martin and Brown 2010), therefore the incorporation of repeated stress and social isolation to our RUS paradigm may have contributed to the decreased basal corticosterone levels in our model as compared to the traditional CUS paradigms. Future studies including earlier timepoints and control groups receiving CUS and SPS are needed to explore the differences between these paradigms and RUS. In contrast to depression, PTSD patients typically show lower plasma cortisol levels compared to healthy controls (Yehuda 2001; Yehuda et al. 1993). While one possible interpretation of our behavioral results is stress induced depression, the blunted HPA axis reactivity is consistent with observations in PTSD and thus the lower plasma corticosterone levels observed at 6 months after RUS suggest that our model has more relevance for PTSD.

The blunted HPA axis response in PTSD is thought to be due to increased negative feedback sensitivity via enhanced GR responsiveness (Hartmann et al. 2012; Schöner et al. 2017; Yehuda et al. 2009). FK506 binding protein 5 (FKBP5) is thought to be a regulator of GR sensitivity. FKBP5 gene knockout mice show increased GR sensitivity resulting in decreased corticosterone levels after restraint stress (Touma et al. 2011). Previous reports have shown that FKBP5 expression levels are reduced in white blood cells and brains of trauma-exposed patients (Levy-Gigi et al. 2013; Yehuda et al. 2009; Young et al. 2015). FKBP5 levels were also reduced in the prefrontal cortex of post-mortem PTSD human subjects (Matthew J Girgenti et al. 2017). In addition, FKBP5 gene polymorphism and epigenetic methylation patterns are associated with GR sensitivity and have also been linked to early-childhood trauma and risk of PTSD (Yehuda et al. 2009; Zovkic and Sweatt 2012). I found the levels of FKBP5 to be reduced in the hypothalamus of RUS mice the 6 months timepoint, supporting a central role for FKBP5 in HPA axis dysregulation in our model (**Figure 2.8D**). These findings together with the reductions in plasma corticosterone and ACTH levels (**Figure 2.8A and 2.8B**) at 6 months after RUS, implicates a dysfunction in the intrinsic homeostatic mechanisms regulating the HPA axis and also highlights the presence of possible molecular abnormalities in the hypothalamus as a contributing factor underlying this dysfunction. Previous reports have also suggested a role for the pituitary gland in dysregulation of HPA axis sensitivity in PTSD patients (Cooper et al. 2017; Ströhle et al. 2008), however a full understanding of the nature of this dysregulation remains elusive. Further chronic studies involving challenging the hypothalamus, pituitary and/or adrenal glands after exposure to our stress exposure paradigm will be needed to explore these possibilities.

HPA axis activation is involved in negative feedback control of the immune response as a natural protective mechanism to prevent excessive inflammation (Gill et al. 2009; Koelsch et al. 2016). The uncontrolled and excessive activation of HPA axis in stress disorders is associated with chronic immune system dysregulation (Gill et al. 2009). Indeed, peripheral inflammation has been reported in a number of psychological disorders such as depression (Deslauriers et al. 2018) and PTSD (Gill et al. 2009; Wang, Caughron, and Young 2017). Specifically, IL1 β levels were elevated in combat PTSD males compared to non-traumatized controls (Spivak et al. 1997). In addition, plasma levels of IFN γ were elevated in PTSD veterans when compared to a healthy control group (Zhou et al. 2014). The low baseline corticosterone levels in our model may underlie a failure of the HPA axis to regulate immune response and therefore explain the elevation in plasma cytokine levels which may contribute towards a primed inflammatory state (Table 1).

Exposure to severe traumatic stress is associated with reduced hippocampus volume (Ahmed-Leitao et al. 2016; Fragkaki et al. 2016; Kim, Pellman, and Kim 2015; McEwen et al. 2016). A recent large-scale multisite neuroimaging study involving 1868 subjects found smaller hippocampi volumes in subjects with PTSD compared to trauma-exposed controls (Logue et al. 2018). In addition, a meta-analysis of magnetic resonance imaging studies of adults with PTSD found hippocampal volume reductions in subjects with PTSD (n=846) compared to traumatized controls (n=624) with the left-hippocampi showing greater reductions (O'Doherty et al. 2015). For the majority of these PTSD subjects, MRI scans were taken more than 10 years since the last trauma (O'Doherty et al. 2015). However, a number of studies have suggested that smaller hippocampal volume is a risk factor for the development of PTSD rather than a trauma consequence (Gilbertson et al. 2002; van Rooij et al. 2015). In animals, chronic restraint stress has been shown

to selectively reduce hippocampal volume in rats (when compared to the pre-stress volume) (Gilbertson et al. 2002). A recent study showed that chronic unpredictable restraint stress or diminished adult neurogenesis can contribute to reduced volumes of both dorsal and ventral hippocampus in rats (Schoenfeld et al. 2017). Our results showed a trend towards reduction in the dorsal hippocampus and a statistically significant reduction in the CA1 of the right dorsal hippocampus 6 months after exposure to RUS. The pyramidal cells of the CA1 subfield of the dorsal hippocampus is involved in processing and retrieval of visuospatial cues or memories (Ji and Maren 2008), and it relays information received from other hippocampal subfields to neocortical areas for higher cognitive processing. The impact of stress on different subfield regions within the hippocampus remains inconclusive. A study on rats found a reduction in the volume of CA1 after two weeks of chronic unpredictable mild stress, while a number of studies found a reduction in the CA3 volume after CUS (McEwen et al. 2016; Morey et al. 2016; Schoenfeld et al. 2017). Impairments within the hippocampal neurocircuitry could contribute towards abnormalities in the encoding, retrieval and processing of memory in PTSD pathobiology; specifically, with regard to the ability to discriminate between safe and aversive contexts, i.e. fear generalization.

Additional experiments in this current model will be required to explore this further using context specific cues that are needed to evaluate fear generalization. In addition, examining the effect of RUS on fear memory recall compared to a control group receiving only fear conditioning is necessary to understand the effect of extinction on recall of contextual and cued fear memories in the current model. These experiments will be essential to understanding the impact of RUS on fear memory recall (i.e. normal vs. abnormal fear memories).

Recent evidence suggests that the ventral hippocampus is involved in emotional memory retrieval and stress reactivity, therefore future studies should also examine the effect of RUS on the volume of the ventral hippocampus (Ritov, Ardi, and Richter-Levin 2014).

The BDNF/TrkB system has been implicated in the pathophysiology of a number of psychiatric disorders, such as schizophrenia (Autry and Monteggia 2012), depression (Autry and Monteggia 2012), and PTSD (Angelucci et al. 2014; Dretsch et al. 2016; Grassi-Oliveira et al. 2008). A single nucleotide polymorphism (SNP) in the BDNF gene, causing a valine to methionine substitution (V66M) is linked to poor working memory, reduced hippocampal volume (Brooks et al. 2014) and alterations in fear extinction in humans (Soliman et al. 2010). In animal studies, V66M was associated with increased anxiety and depression-like behavior (Chen et al. 2007). In a rat model of PTSD, exposure to psychosocial stress was linked long-term epigenetic changes in hippocampal BDNF DNA (Roth et al. 2011). In addition, several types of stressors, including restraint stress, social defeat, and electric foot shocks, result in a decrease in BDNF levels in the hippocampus (Lee and Kim 2010; Yu and Chen 2011). Consistent with our data showing abnormal passive stress coping behavior in animals exposed to RUS, BDNF levels were significantly reduced in the hippocampus of mice exposed to stress (**Figure 2.10D**). Given that I did not observe any impairments in hippocampal-related tasks, such as spatial learning, and recall of reference memory (**Figure 2.7**), and taken together, the lower BDNF levels and the reduced CA1 volume observed herein, suggest that exposure to RUS interferes with a unique yet unknown function of the hippocampus in regulating aspects of fear memory expression and alterations in stress coping strategies (**Figure 2.4**). Regions such as the prefrontal cortex, amygdala and hypothalamus which are associated with these

functions, can receive and project their connections to the hippocampus, and therefore future studies investigating this complex neurocircuitry may help elucidate the role played by this critical brain region in PTSD.

Acute stress exposure and fear learning are associated with immediate (mins to hrs.) increase in amygdala activity as detected by imaging studies, and neuroplasticity (increased NMDA receptors and downstream synaptic signaling partners) (Balu et al. 2017; Yasmin et al. 2016). I did not examine the immediate effects (mins to hrs.) of stress on amygdala activity, however, the reduced expression levels of NMDA receptors and related neuroplasticity markers observed at the acute timepoint (**Figure 2.12**) may suggest the presence of compensatory mechanisms to the initial hyperactivity in the amygdala neurocircuitry. The hypothalamus is rich in neurons that secrete numerous neuropeptides that regulate several autonomic functions implicated in regulating neurocircuitry pathways involved in mood and stress management. I conducted molecular profiling of regulatory neuropeptides within the hypothalamus and observed a significant increase in Orexin-A levels at 6 months after stress (**Figure 2.9**). Orexin-A is a key regulator of the sleep/wakefulness cycle(Bee 2009; Tsujino and Sakurai 2013). Sleep disturbance and occurrence of traumatic nightmares is a common feature of PTSD in humans. A number of studies have drawn a critical link between Orexin system dysregulation and stress/anxiety disorders(Johnson et al. 2010; P. L. Johnson et al. 2012). Orexins are also believed to be an emerging target for treatment of PTSD (Krystal et al. 2017), however, further examination of Orexin signaling and circuitry is needed to understand their role in PTSD pathophysiology. This finding suggests the suitability of this model as a platform to explore the role of Orexins in stress disorders.

Retesting animals using very similar behavioral test batteries is one of the limitations in this study since most of the behavioral tests are sensitive to retesting. Future experiments should utilize different behavioral tools to examine the same behavioral at different timepoints domain. For example, the Open Field, EPM, and the light-dark box can be used to examine anxiety-like behavior at different time points instead of examining all animals in the Open field multiple times. The anxiety scores of each particular animal can then be correlated with different molecular markers and also can be used to evaluate the progression of stress-related behavior over time.

Using the t-test to evaluate statistical differences between control and stressed animals at each timepoint independently is another limitation of the current study. To correct for multiple comparisons at different timepoints, a posthoc test should have been used after the analysis. These procedures would have granted better control of the false discovery rate. However, since the results presented in this chapter are primarily exploratory, I used a less stringent test (t-test) to avoid overlooking any significant discoveries.

Chapter 3. Impact of r-mTBI on behavioral and hippocampal deficits in a mouse model of chronic PTSD

3. 1 Introduction

The consequences of post-traumatic stress disorder (PTSD) and mild traumatic brain injury (mTBI) have become a prime research focus in the past decade. The issue received attention with the increasing number of returning veterans from Iraq and Afghanistan experiencing one or both conditions. The prevalence of PTSD in soldiers returning from Operation Iraqi Freedom (OIF) and Operation Enduring Freedom (OEF) ranges from 4% to 17% for those in the United States (Hines et al. 2014; Hoge et al. 2008). In addition, 18% of returning veterans suffered mild TBI largely due to exposures to blast waves from improvised explosive devices (Hoge et al. 2008).

While the number of PTSD and TBI patients is still growing, many questions concerning comorbid PTSD and TBI remain unanswered. For example, would a history of TBI increase the likelihood of developing PTSD after exposure to a traumatic event? Does the timing of TBI relative to the traumatic event affect PTSD-related outcomes? The complexity of these questions increases when considering the different severities of TBI (i.e. mild, moderate or severe). Attempting to answer these questions using human studies is challenging as there are several variables to account for, and because of the clinical heterogeneity and overlapping symptoms of mTBI and PTSD. Therefore, developing a preclinical model that captures the complexity of both conditions, either individually or comorbidly, is crucial in order to understand the underlying neurobiological mechanisms and establish better healthcare strategies.

In our TBI research, we have focused on the chronic effects of repetitive mild TBI (r-mTBI), utilizing a mild injury delivered on the midline of a closed skull, administered 5 times over a 9 day period (Mouzon et al. 2014). With this model we have demonstrated that, in contrast to single mTBI, repetitive mTBI was associated with ‘chronic’ spatial memory deficits, axonal injury, and glial activation long after the last injury (Mouzon et al. 2014, 2012). Because the trauma-related symptoms are persistent in human PTSD, the presence of long-lasting behavioral alterations in a PTSD mouse model is crucial for its validity. As demonstrated in Chapter 2, I have shown that exposure to a repeated unpredictable stress (RUS) was associated with several PTSD-like phenotypes including passive stress-coping behavior and long-lasting abnormalities in the HPA axis and the hippocampus. Since both the r-mTBI and RUS models were associated with chronic outcomes, they represent a suitable platform in which to investigate comorbid PTSD and mTBI.

I first conducted a preliminary study to examine the impact of their combined exposure on behavioral outcomes. The integration of the two paradigms was achieved by administering a single mTBI one hour after the foot-shock on five different days. Interestingly, I found that r-mTBI abrogated the stress-related phenotype in the cued fear memory and the forced swim tests (Experiment 1). This preliminary study was developed to resemble a single military deployment, where soldiers are exposed to multiple repeated stressful events in addition to concussions (mTBI).

Given the moderate effects observed with the RUS paradigm on chronic measures at 6 months post-exposure in chapter 2, these findings further led to the idea that a dose response of stressful exposures (and injuries) experienced by individuals who are exposed to multiple and repeated traumatic events on separate occasions over their life time, may

elicit a worse outcome. This is demonstrated by epidemiological studies showing a positive correlation between number of deployments and higher risk for PTSD diagnosis (Kline et al. 2010) and also increased risk for mental health disorders in civilian population exposed to repeated multiple non-combat trauma sources (Tanskanen et al. 2004).

To that end, in this chapter, I explore the consequences of double exposure to our RUS and r-mTBI procedures on behavioral and biological outcome. I hypothesized that both stress-dependent and r-mTBI-dependent phenotypes will be more prominent after repeated exposure compared to a single exposure paradigm. The comprehensive characterization of the long-lasting behavioral and neurobiological outcomes of repeated RUS and r-mTBI exposure that will be presented in this chapter, are particularly relevant to active duty soldiers receiving several combat deployments and multiple traumatic events. I anticipate that this model will be a good platform to untangle the complex comorbid pathophysiology in the heterogeneous population of PTSD patients who experience different history of traumatic events and enable further exploration of new treatment strategies to address comorbid PTSD and repetitive mTBI.

3. 2 Materials and Methods

Animals

12-week-old C57BL/6 male mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed in standard cages under a 12-h light/12-h dark schedule at ambient temperature. All procedures were performed in accordance with OLAW guidelines under a protocol approved by the Roskamp Institute IACUC. Animals were randomly assigned to four groups: Controls, Stress, r-mTBI or Stress+r-mTBI (N=15/group).

Experiment 1. The 21-day repeated unpredictable stress (RUS) and repetitive mild traumatic brain injury (r-mTBI) paradigm.

Our repeated unpredictable stress (RUS) paradigm was implemented as described previously and as depicted in **Figure 3.1**. In brief, 12-week-old mice received 21 days of RUS involving (i) unstable social housing with a different mouse every day, (ii) 30 minutes of unpredictable exposure to a predator odor (TMT) under restraint on 10 different days, and (iii) five inescapable foot-shocks. Animals assigned to the stress+r-mTBI group received (iv) a mild traumatic brain injury an hour after exposure to each foot-shock, while animals in the r-mTBI group received the five mTBIs only and no foot-shocks. The one-hour delay was introduced to allow for consolidation of fear memory. Closed head injuries were conducted using our previously established model involving a 1mm depth impact delivered to the closed skull using a 5mm flat tip electromagnetic stereotaxic impactor at a velocity of 5m/s with a dwell time of 200 milliseconds (Mouzon et al. 2012). Mice were anesthetized using 1.5L/min of oxygen and 3% isoflurane and maintained under anesthesia

through a nose cone throughout the injury procedure. Foot-shocks and head injuries were administered at 48-72 hour intervals to replicate our previous characterization of the r-mTBI model. Control and r-mTBI-only animals were exposed to the fear-conditioning chamber for the same frequency and duration as the stressed mice, without administration of foot-shocks or auditory cues, and were never restrained or exposed to TMT.

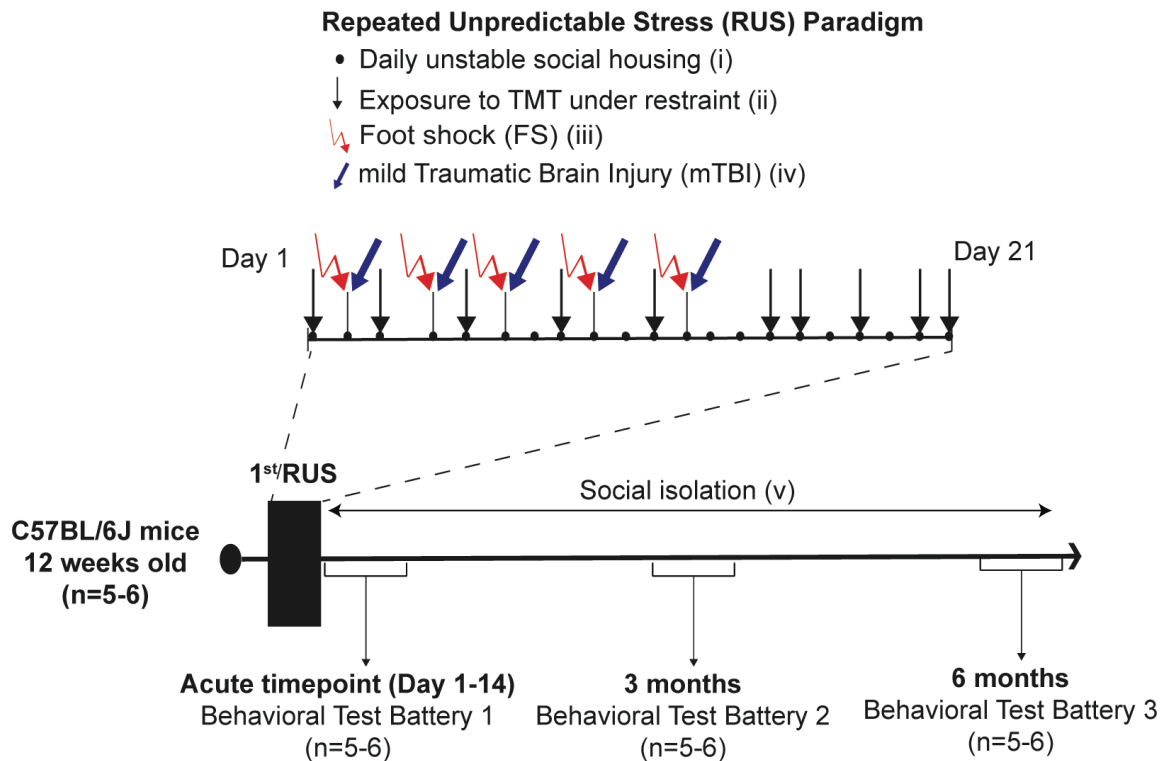


Figure 3.1. Study timeline and experimental procedures in Experiment 1. The stress paradigm involved 21 days of daily unstable social housing (i), unpredictable repetitive exposures to predator odor (Fox urine, TMT), while under restraint for 30 mins (ii), and five repeated inescapable foot-shocks (iii). Animals receiving repeated mild traumatic brain injury (r-mTBI) in addition to stress (stress+r-mTBI group) were exposed to (iv) five closed head injuries an hour after exposure to inescapable foot-shocks. Closed head injuries were conducted using our previously established model involving a 1mm depth impact to the closed skull using a 5mm flat tip electromagnetic impactor at a velocity of 5m/s with a dwell time of 200ms. All animals in the stress groups were singly housed (Social isolation) after RUS until the end of the study (v).

Experiment 1. Behavioral testing

All behavioral experiments were conducted as described in the materials and methods section in Chapter 2. I did not perform any molecular studies on the cohort of animals used in Experiment 1. Control and stress-only animals examined in this study were a subset of the animals tested previously in Chapter 2. The behavioral test batteries are identical to the ones described in Chapter 2 (**Figure 2.2**).

Experiment 2. The 21-day repeated unpredictable stress (RUS) and repetitive mild traumatic brain injury (r-mTBI) paradigm.

The same RUS and r-mTBI procedures described in Experiment 1 were repeated again 3 months after the first RUS (i.e. when animals were 27 weeks old) as depicted in **Figure 3.2**. Animals in the stress and stress+r-mTBI groups were housed individually after RUS procedure, whereas animals in the control and r-mTBI groups were housed in groups of 2-3 mice throughout the study.

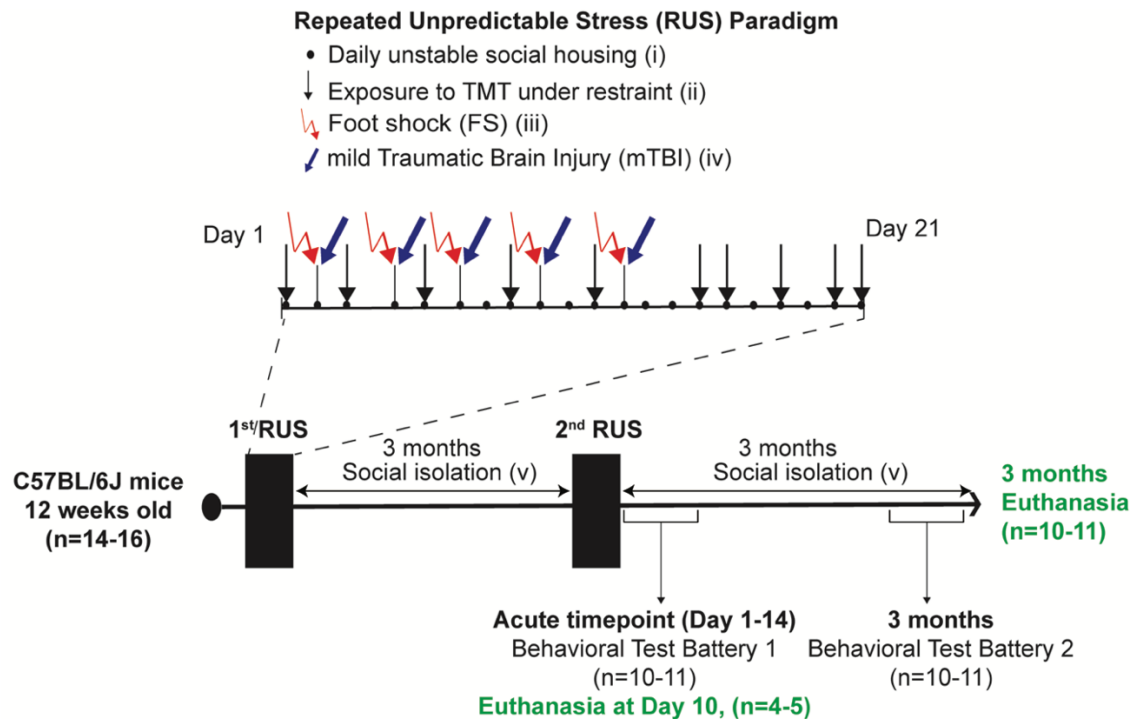


Figure 3.2. Study timeline and experimental procedures in Experiment 2. The RUS procedure was implemented as described in Experiment 1. C57BL/6J mice (12-week-old) were exposed to the RUS paradigm at 3 months and at 6 months of age. A battery of behavioral testing was conducted at an acute timepoint (Days 1-19) and again at 3 months after the second RUS. Brain tissue and plasma were collected at the acute timepoint and at the 3-month timepoint.

A separate group of animals (n=4-5 per group) were euthanized at 10 days after the second RUS (acute timepoint) for molecular studies. To increase the animal number of animals in the anxiety tests, these animals received the Open Field test and the Elevated Plus Maze only once at the acute timepoints with no additional testing. The remaining animals (10-11) per group received a behavioral testing battery beginning at day 1 after the second RUS (acute timepoint) and then were retested using a similar battery at 3 months after the second RUS. The timeline of the behavioral test batteries is shown in **Figure 3.3**. All animals (10-11) were euthanized one week after the last behavioral experiment for molecular studies (3 months after the second RUS).

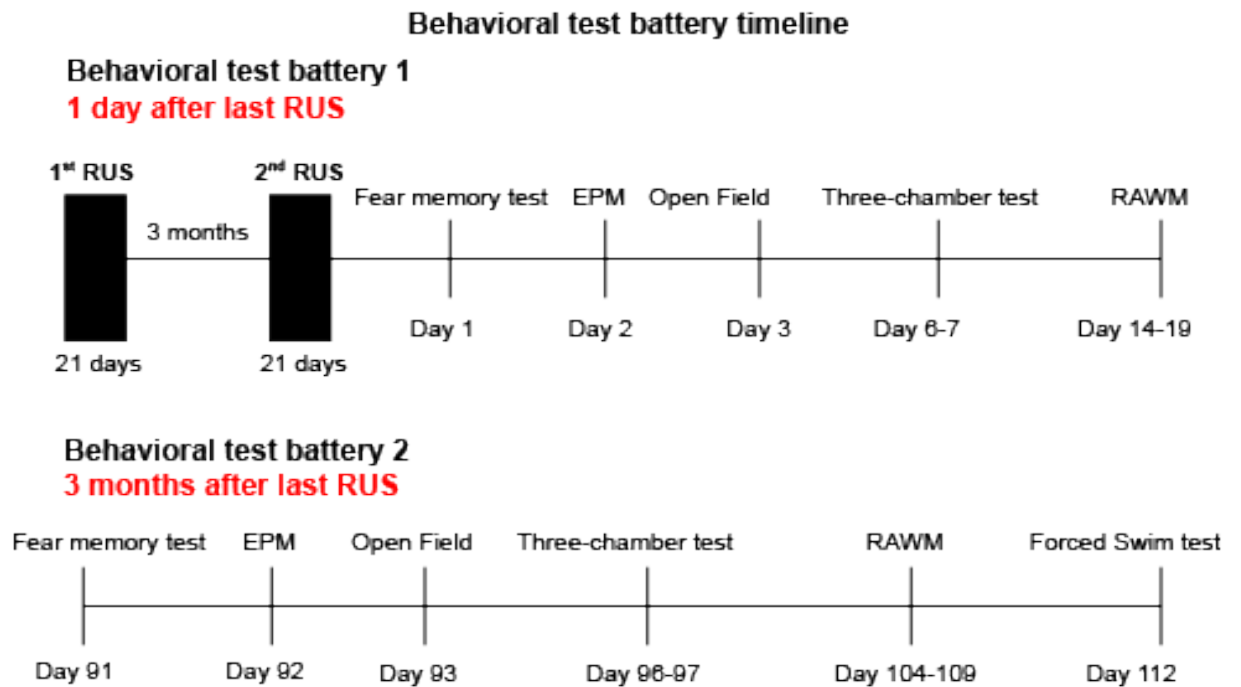


Figure 3.3. Timeline of behavioral test batteries. The same animals were retested at behavioral test battery 2.

Experiment 2. Fear conditioning and behavioral testing

All fear conditioning and behavioral experiments were conducted as described in the materials and methods section in Chapter 2.

Experiment 2. Blood collection and ex-vivo LPS stimulation of whole blood

To obtain blood samples, animals were anesthetized with isoflurane, and approximately 500 μ L of blood were collected into heparin tubes by cardiac puncture immediately prior to euthanasia. 400 μ L of blood from each animal were kept aside for the

ex-vivo LPS stimulation studies. The remaining blood was centrifuged at 3000g for 3 min, and plasma samples (clear supernatant fraction) were flash frozen in liquid nitrogen and stored at -80°C . For the ex-vivo LPS challenge, 400 μL blood samples from each animal were divided into two 200 μL samples and incubated with either RPMI (1% glutamine, 1% pyruvate, 1% garamycin) or RPMI containing 1 $\mu\text{g}/\text{mL}$ LPS at 37°C . After 6 hours at 37°C , all blood samples were centrifuged for 3 min at 3000g for plasma collection.

Experiment 2. Enzyme-linked immunosorbent assay (ELISA) and Multiplex ELISA

Commercially available ELISA kits were used to measure the concentration of several plasma markers as follows; the neuropeptide Y (NPY) ELISA kit was purchased from Life-Span biosciences (Seattle, Washington), the norepinephrine ELISA kit was purchased from Eagle biosciences (Amherst, New Hampshire), and the corticosterone ELISA kit was purchased from Arbor Assays (Ann Arbor, Michigan). Tissue brain-derived neurotrophic factor (BDNF) levels were measured using an ELISA from Boster Bio (Pleasanton, California). All ELISA kits were used as per manufacturers' instructions. Brain and plasma cytokine levels were measured using the MSD proinflammatory Panel I for the following cytokines: interferon γ (IFN- γ), interleukin (IL)-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, and tumor necrosis factor α (TNF α) (MesoScale Discovery, Gaithersburg, Maryland). A single plasma sample (12.5 μL) from each mouse was used for the detection of plasma cytokines, while 25 μL of hippocampal homogenate in M-PER protein extraction reagent (~ 8 mg/mL total protein) was used for the measurements of hippocampal cytokine levels.

Experiment 2. Brain tissue preparation and western blotting

Brains from 4-6 animals per group were used for biochemical analysis while the brains from the remaining animals were used for immunohistochemical experiments. Western blot experiments were conducted as described previously (Ojo et al. 2016). In brief, following transcardial perfusion by gravity drip with phosphate-buffered saline (PBS), brain tissue was collected and the hippocampus and hypothalamus were dissected and frozen immediately in liquid nitrogen and kept at -80°C . For western blotting analyses, the hypothalamus or hippocampi from both hemispheres were homogenized in 200 μL of M-PER protein extraction reagent containing proteinase and phosphatase inhibitors (ThermoFisher) using a probe sonicator. Samples were sonicated with a 2 s pulse followed by incubation on ice for 10 s, and this process was repeated 3 times. Homogenized samples were centrifugated at 15,000g for 10 minutes (min) followed by collection of the supernatants. Diluted supernatant fractions (2mg/mL) were then mixed with Laemmli buffer (Bio-Rad) containing dithiothreitol (DTT) and denatured by boiling at 99°C . Samples were then subsequently resolved on 4% to 15% gradient polyacrylamide criterion gels (Bio-Rad). After electrotransferring to polyvinylidene difluoride membranes, membranes were blocked in 5% milk made in Tris-buffered saline and subsequently incubated with different primary antibodies overnight (**Table 2.1**). After three washing steps using TBST, membranes were probed with horseradish peroxidase-linked secondary antibodies (**Table 2.1**). Signal intensity ratios were quantified by chemiluminescence imaging with the ChemiDocTM XRS (Bio-Rad) with Anti-GAPDH antibody used as a housekeeping protein.

Experiment 2. Golgi-Cox staining and Immunohistochemistry

5-6 animals per group were used in Golgi-Cox staining and immunohistochemistry experiments. At 3 months after the second RUS exposure, mice were anesthetized with 3% isoflurane and transcardially perfused with PBS solution. Right brain hemispheres were extracted and immediately processed according to FD Rapid Golgi stain Kit instructions (FD NeuroTechnologies, Baltimore, MD), while the other hemispheres (left side) were fixed in 4% paraformaldehyde for 24–48 hours followed by embedding in paraffin for subsequent immunohistochemistry (IHC) experiments.

For the Golgi experiments, 100 μ m sections of impregnated tissue were cut using a cryostat at -20°C, mounted onto positively charged glass slides (Fisher, Superfrost Plus, Pittsburgh, Pennsylvania) and analyzed using an Olympus BX63 upright microscope at 100x magnification. The following three criteria were met by a dendrite to be chosen for spine density analysis: 1) it had to be emerging either from an apical neuron in the hippocampal CA1 region or layer II/III in the frontal cortex, 2) the neuron had to be fully stained including the cell body with no cuts in the axons, and 3) the origin of the dendrite should not be obscured by any nearby overlapping neurons.

For IHC experiments, a series of 6 μ m-thick sagittal sections were cut throughout the extent of the cortex and hippocampus guided by known bregma coordinates using a microtome (2030 Biocut, Reichert/Leica, Buffalo Grove, Illinois). Cut sections were mounted onto positively charged glass slides (Fisher, Superfrost Plus, Pittsburgh, Pennsylvania). Before the IHC procedure, all sections were deparaffinized in xylene and rehydrated in a gradient of ethanol solutions of decreasing concentrations. Sections were then rinsed in distilled water and subsequently incubated at room temperature in a solution

of hydrogen peroxide (3% in water) for 15 minutes to block endogenous peroxidase activity. Antigen retrieval was necessary for Iba1 staining and included treatment with boiling citrate buffer solution (pH 6) for 8 minutes in the microwave. Following antigen retrieval, sections were blocked for 30 minutes to 1 hour in normal blocking serum, using the same serum in which the secondary antibody was raised. Next, sections were immunostained in batches with primary antibodies diluted in supersensitive wash buffer (BioGenex, Fremont, CA). The primary antibodies used are listed in Supplementary Table 1. After overnight incubation at 4°C, sections were washed in PBS and then incubated with the appropriate secondary antibody (Vectastain Elite ABC Kit, Vector Laboratories) at room temperature for 30 minutes to 1 hour, depending on the specific requirement of the antibody protocol. After rinsing in water, sections were incubated with avidin-biotin horseradish peroxidase or alkaline phosphatase enzyme solution (Vector Laboratories) for 30 minutes at room temperature. Immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB) peroxidase solution (0.05% DAB - 0.015% H₂O₂ in 0.01M PBS, pH 7.2) (Vector Laboratories). Development with the chromogen was timed and applied consistently across batches to limit technical variability before progressing to quantitative image analysis. Reactions were terminated by rinsing sections in distilled water. Sections were counterstained with hematoxylin, dehydrated through a gradient of ethanol of increasing concentrations, cleared in xylene, and coverslipped with permanent mounting medium. Immunoreacted sections were viewed using a motorized Olympus BX63 upright microscope and photographs were taken using the high-resolution DP72 color digital camera.

Experiment 2. Image Analysis

Immunoreactivity for glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) was measured by quantitative image analysis (optical segmentation) by an observer blinded to experimental grouping. Multiple regions of interest were analyzed in standardized fashion for each cell marker/antibody. A survey of immunostained tissue sections was first performed independently to verify specific immunoreactivity that was subsequently progressed to quantitative image analysis. Briefly, nonoverlapping red, green, blue (RGB) images were digitally captured randomly within the defined areas from each section (comprising an average of 5 sections per animal for each marker), providing a systematic survey of each region of interest for each animal within a group. A minimum of 10 microscopic fields (60x magnification) were analyzed per region per animal. The total microscopic field analyzed for each brain region was an area of 3.5 mm² per animal. Immunoreactive profiles that were optically segmented were analyzed using CellSens morphometric image analysis software (Olympus, Center Valley, Pennsylvania). A semiautomated RGB histogram-based protocol (specified in the image analysis program) was used to determine the optimal segmentation (threshold setting) for immunoreactivity for each antibody. Immunoreactive profiles discriminated in this manner were used to determine the specific immunoreactive percentage area. Data were separately plotted as the mean percentage area of immunoreactivity per field (% area) \pm SE for each region and grouping.

Statistical Analysis

The choice of the appropriate statistical test was made after assessment of normal distribution using the Shapiro-Wilk normality test. Repeated Measures-Two-way ANOVA was used to examine statistical significance in bodyweight measurements, fear memory testing, EPM, Open Field, RAWM training trials, Immunohistochemical data for GFAP and Iba1, and cytokine levels in the ex-vivo LPS challenge. One-Way ANOVA was used to analyze total immobility time in the forced swim test, RAWM one-day trials, profiling of plasma and brain markers using ELISA and western blotting. All ANOVA experiments were followed by pairwise comparisons with correction for multiple comparisons using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (BKY) to control the false discovery rate. The three-chamber test was analyzed using one-sample t-tests. All analyses were performed with Graph Pad prism version 7.0 statistical software (La Jolla, California). Grubbs' test was applied to identify statistical outliers.

3. 3 Results

Experiment 1. Effect of single exposure to RUS and/or r-mTBI on fear memory recall

Data for control and stressed animals in this section has been previously used in to generate the data presented in Chapter 2. Stress-only animals showed a significant increase in their freezing responses to both the context (**Figure 3.4A**) and the auditory cue (**Figure 3.4D**) compared to control animals when examined at the acute timepoint. However, only the response to the auditory cue persisted at 3 months and 6 months after RUS (**Figure 3.4E and 3.4F**). Exposure to r-mTBI abrogated contextual fear memory observed at the acute timepoint (**Figure 3.4A**). On the other hand, no significant r-mTBI effect is observed with cued fear memory at this time point (**Figure 3.4D**). Interestingly, r-mTBI inhibited the retrieval of the cued fear memory response at 3 months and 6 months after RUS (**Figure 3.4E and 3.4F**).

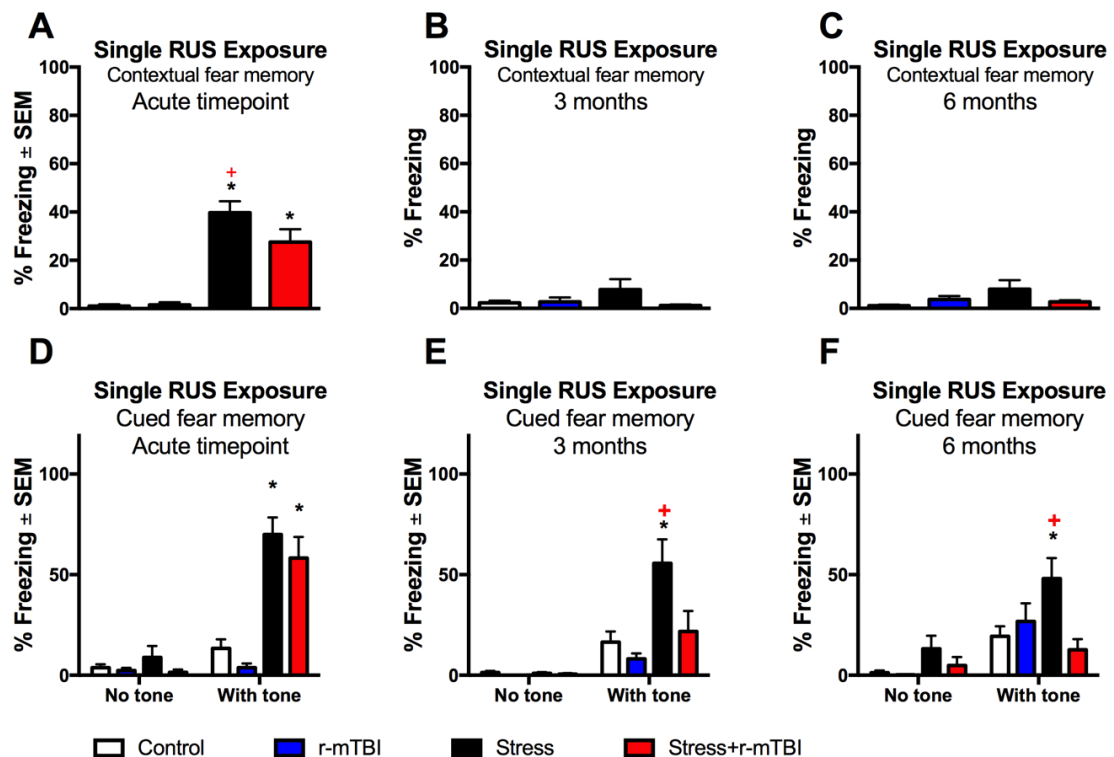


Figure 3.4. Recall of contextual and cued fear memory after single exposure to RUS and/or r-mTBI. Animals in the stress and stress+r-mTBI groups showed a significant increase in their % of freezing to the context compared to control animals only at the acute timepoint (A-C). Stress+r-mTBI animals showed reduced freezing times in the fear conditioning context relative to the stress group only at the acute timepoint (A). In the cued fear memory test, no difference between stress-only and stress+r-mTBI groups was observed at acute timepoint (D). However, at the 3-months (E) and 6-months (F) timepoints, stress+r-mTBI animals showed reduced freezing times relative to the stress-only animals in the cued fear memory test. Data in A-C were analyzed using One- Way ANOVA, while data in D-F were analyzed with Two-way ANOVA. (n=5-6)

Experiment 1. Effect of single exposure to RUS and/or r-mTBI on anxiety-like behavior

In order to test anxiety related behavior in our animal groups, we conducted the elevated plus maze and the open field tests (**Figure 3.5**). In the open field test, stress, r-mTBI only and stress+r-mTBI groups had fewer mean numbers of entries to the center zone compared to the control mice at the acute and 3-month timepoints (**Figure 3.5A and 3.5B**), but this observation was not statistically significant. In the EPM test at the acute timepoint, mice receiving r-mTBI only, showed a significant increase in the mean number of entries to the open arms, consistent with a disinhibitory like behavior (**Figure 3.5D**). On the contrary, stress and stress+r-mTBI animals' examination of the open arms was similar to the control group at this timepoint (**Figure 3.5**). No significant changes were observed in the EPM test at 3 months or 6 months after RUS (**Figure 3.5E and 3.5F**)

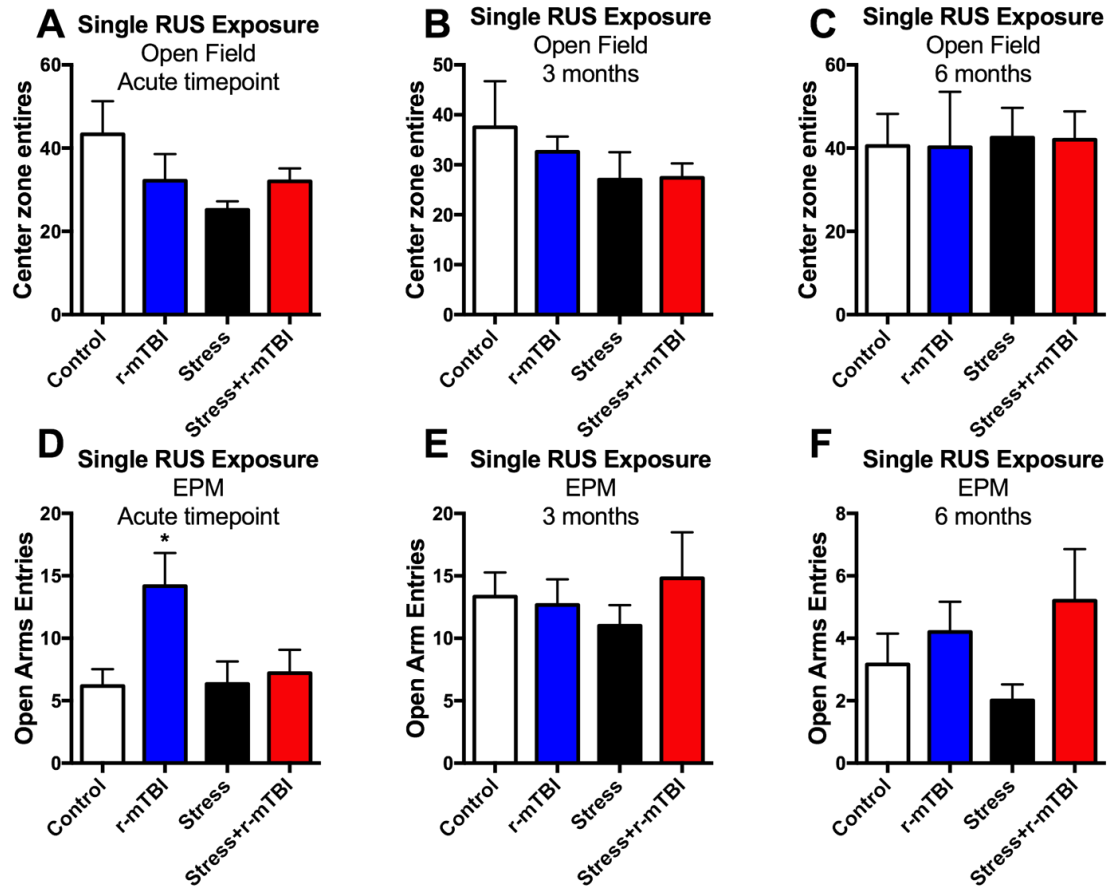


Figure 3.5. The performance of each study group in the Open Field and the EPM. Panels A, B, and C shows the mean number of entries to the center zone in an open field at the acute time point, 3 months, and 6 months after RUS, respectively. The performance of each study group in the EPM at acute time point, 3 months, and 6 months after RUS is depicted in panel D, E, and F, respectively. There was an increase in EPM open arm entries in the r-mTBI group compared to controls at the acute timepoint (D). The EPM test was performed in bright-light conditions at the acute timepoint and in dark (1Lux) conditions at 3, and 6 months after RUS. Data were analyzed using with one-way ANOVA test (n=5-6).

Experiment 1. Effect of single exposure to RUS and/or r-mTBI on spatial memory

The performance of each group during training sessions in a radial arm water maze (RAWM) is depicted in **Figure 3.6**. Mice received 3 sets of acquisition training sessions.

The first set started 3-weeks after RUS followed by a second set at 3 months after RUS and

a third set at 6 months after RUS. No significant learning impairments were observed for any of the groups at acute, 3-month or 6-month timepoints (**Figure 3.6A-C**). Animals receiving r-mTBI-only showed increased memory errors at 3-month and 6 months timepoints when compared to control mice at the last day trial (RM-Two-Way ANOVA: interaction $F_{(12, 60)} = 1.19$, $p > 0.05$, main stress and/or r-mTBI effect $F_{(3, 60)} = 8.8$, $p < 0.003$ with correction for multiple comparisons, **Figure 3.6B**) and (RM-Two-Way ANOVA: interaction $F_{(6, 45)} = 1.4$, $p > 0.05$, main stress and/or r-mTBI effect $F_{(3, 45)} = 3.56$, $p < 0.02$ with correction for multiple comparisons, **Figure 3.6C**).

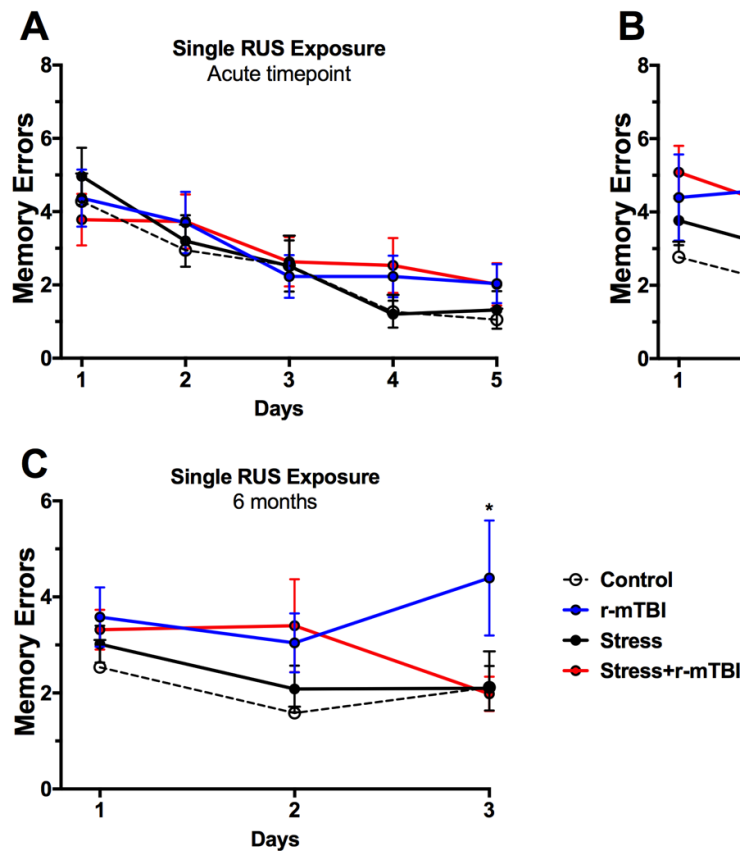


Figure 3.6. Effect of single exposure to RUS and/or r-mTBI on the performance of each group in the Radial Arm Water Maze. No significant differences between groups were observed at the acute timepoints. In the last day of training at 3 months and 6 months after RUS, r-mTBI animals showed increased mean memory errors relative to all other groups. Data were analyzed using repeated measures RM-Two-Way ANOVA.

Experiment 1. Effect of single exposure to RUS and/or r-mTBI on stress-coping strategies

At 6 months after RUS, animals in the stress-only group showed increased immobility time relative to stress+r-mTBI group in the Forced Swim Test (FST) (One-way ANOVA, $F(3, 17) = 4.02$, $p = 0.025$, with correction for multiple comparisons, **Figure 3.7**).

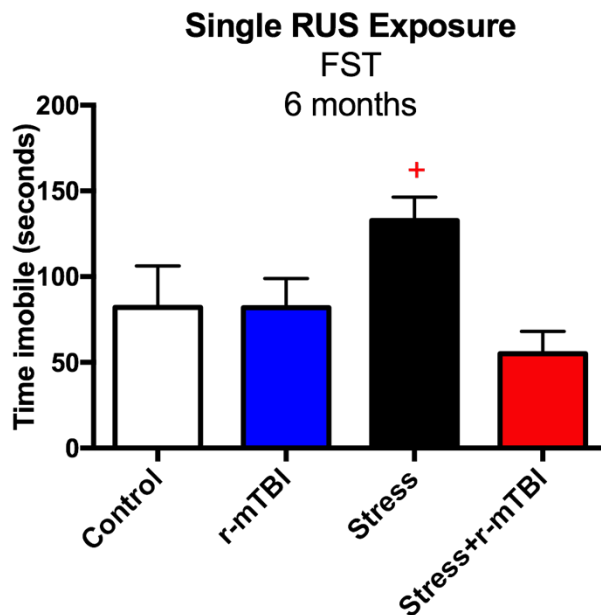


Figure 3.7. Effect of single exposure to RUS and/or r-mTBI on the performance of each group in the Forced Swim Test. Stress animals showed increased immobility time in the forced swim test relative to the stress+r-mTBI group at 6 months after RUS. Data were analyzed using with one-way ANOVA test (n=5-6).

Experiment 2. Repeated exposure to stress and/or mTBI results in acute bodyweight loss.

I have previously shown that a single exposure to the RUS paradigm results in a reduction in normal bodyweight gain in young adult mice (Algamil et al. 2018). In experiment 2, I examined the effect of repeated exposure to stress and mTBI on bodyweight. During the first RUS exposure body weight was only affected by stress

alone, as evidenced by reduced weight gain in the stress and stress+r-mTBI groups in the first 3 weeks of stress compared to controls (RM-ANOVA: interaction $F_{(9, 102)} = 2.835$, $p < 0.005$, with correction for multiple comparisons, **Figure 3.8A**). No significant r-mTBI effects on bodyweight were observed during the first RUS (**Figure 3.8A**). Stress and/or r-mTBI procedures were repeated again 3 months after initial stress (second RUS), during which animals in the r-mTBI, stress and stress+r-mTBI groups showed significant weight loss at days 14 and 21 of RUS when compared to the control group (RM-ANOVA: interaction $F_{(9, 111)} = 3.816$, $p < 0.0001$, with correction for multiple comparisons, **Figure 3.8B**). The TBI-related weight loss during the second RUS was worsened by stress as evidenced by increased weight loss in the stress+r-mTBI group relative to the r-mTBI group (**Figure 3.8B**). The changes in bodyweight in all groups were driven by an intense decrease in their growth rate during the first week of RUS or r-mTBI (RM-ANOVA: interaction $F_{(9, 105)} = 3.816$, $p = 0.0003$, with correction for multiple comparisons, **Figure 3.8C and 3.8D**).

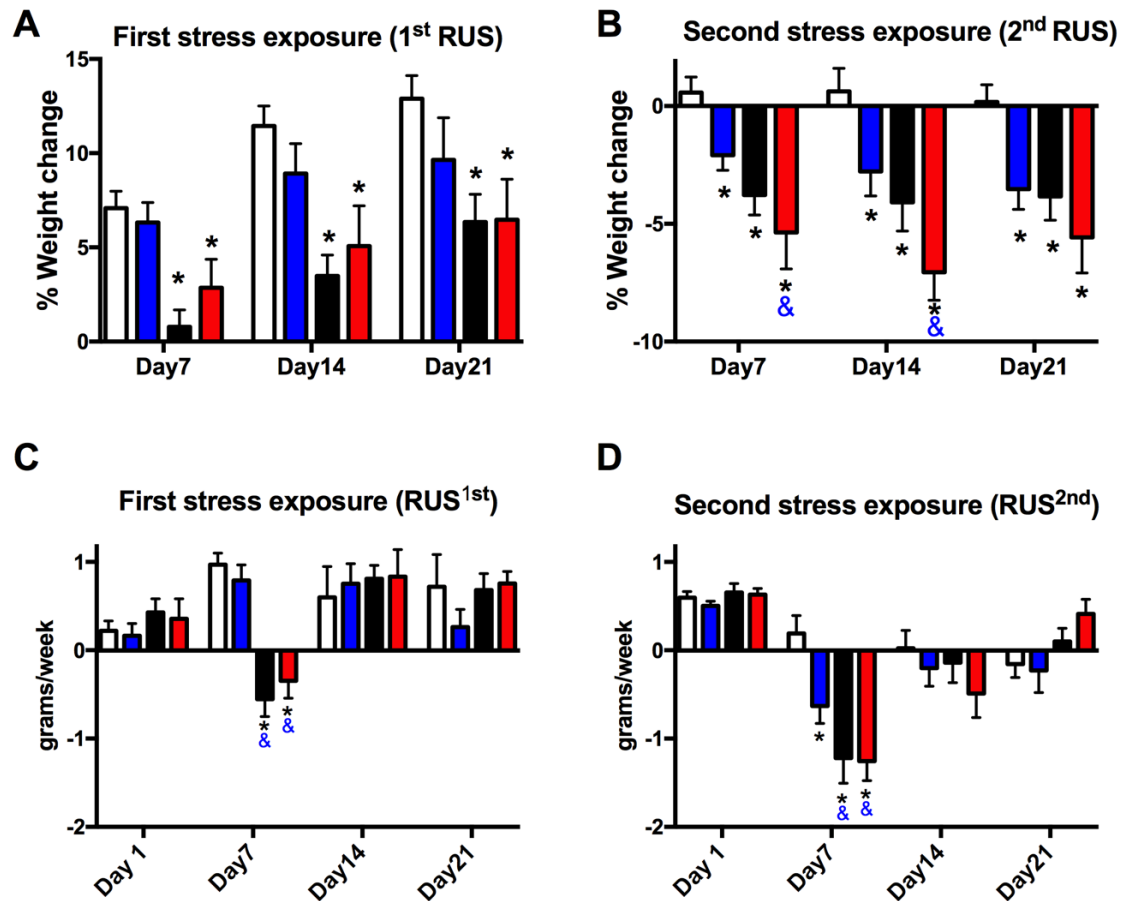


Figure 3.8. Effect of double exposure to RUS on bodyweight gain. (A) During the first exposure to stress and/or r-mTBI (1st RUS), animals in the stress and stress+r-mTBI groups showed a significant reduction in their bodyweight gain throughout the 21 days of stress. Stress and/or r-mTBI procedures were repeated 3 months after initial stress (2nd RUS). (B) During the second RUS, all treatment groups showed significant weight loss at days 7, 14 and 21 of the 2nd RUS compared to the control group. Animals in the stress+r-mTBI group showed a greater weight loss when compared to the r-mTBI-only group at days 7 and 14 of the 2nd RUS (B). Changes in growth rate after the first and second RUS are depicted in panels C and D, respectively. Data in A-D were analyzed using repeated measures Two-Way ANOVA followed by two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (BKY) (n = 9-11). Statistically significant discoveries versus the control group are denoted by “*”, while statistically significant discoveries versus the r-mTBI group are denoted by “&”.

Experiment 2. Effect of r-mTBI on overall locomotion and recall of fear memory.

I next examined the effect of r-mTBI on several stress-related behaviors (Recall of fear memory, and stress-coping strategies) at the acute timepoint and at 3 months after the second RUS (**Figure 3.9**). All animals receiving RUS, independent of r-mTBI, showed a significant increase in their freezing (immobility time) when placed in the same trauma context at the acute and 3-month timepoints (RM-Two-Way ANOVA: interaction $F(3, 39) = 0.5347$, $p > 0.05$, main stress/r-mTBI effect $F(3, 39) = 23.75$, $p < 0.0001$ with correction for multiple comparisons, **Figure 3.9A**). In the cued fear memory test at the acute timepoint, stress and stress+r-mTBI groups showed similarly increased freezing responses to the cue compared to their respective control groups in the total test duration (Two-way ANOVA, interaction $F(3, 78) = 16.22$, $p < 0.0001$, **Figure 3.9B**), and in the 30 s time-bin analysis (RM-Two-Way ANOVA: interaction $F(15, 228) = 0.4668$, $p > 0.05$, main stress/r-mTBI effect $F(15, 228) = 86.23$, $p < 0.0001$ with correction for multiple comparisons, **Figure 3.9D**). However, at the 3-month timepoint, the stress+r-mTBI group showed reduced immobility time relative to the stress-only group in the cued fear memory test (Two-way ANOVA, interaction $F(3, 78) = 6.57$, $p < 0.0011$, with correction for multiple comparisons, **Figure 3.9C**). Interestingly, stress+r-mTBI animals showed an increased average immobility time relative to controls in the contextual fear memory test, but not in the 3-min cued fear memory test at the 3-month timepoint. A decreased startle response in r-mTBI animals at 3 months after the last injury is a possible explanation to this observation. However, a closer inspection of the freezing behavior in 30 s time-bins (**Figure 3.9E**) shows that stress+r-mTBI and stress-only animals exhibited a similar increase in their immobility time during the first 60 s of the test (RM-Two-Way ANOVA: interaction $F(15, 234) = 1.53$, $p > 0.05$, main stress/r-mTBI effect $F(15, 234) = 36.63$, $p < 0.0001$ with

correction for multiple comparisons, **Figure 3.9E**). Therefore, stress+r-mTBI animals do not appear to show a reduced startle response relative to the stress-only group. The immobility time parameter relies on animals' inactivity or lack of body movement to score freezing. To examine the overall locomotor activity, I first checked the total distance travelled by different study groups in a safe new context and in the absence of any stress-related cues. As depicted in **Figure 3.9F**, stress+r-mTBI and r-mTBI-only animals travelled longer distances in the 3-min-long test relative to the control animals and stress-only animals respectively (RM-Two-Way ANOVA: interaction $F(3, 38) = 0.518$, $p > 0.05$, main stress/r-mTBI effect $F(3, 38) = 8.9$, $p < 0.0001$ with correction for multiple comparisons, **Figure 3.9F**). Remarkably, stress-only animals travelled a shorter distance relative to the controls at the acute timepoint (**Figure 3.9F**), suggesting fear generalization or anxiety-like behavior shortly after RUS exposure.

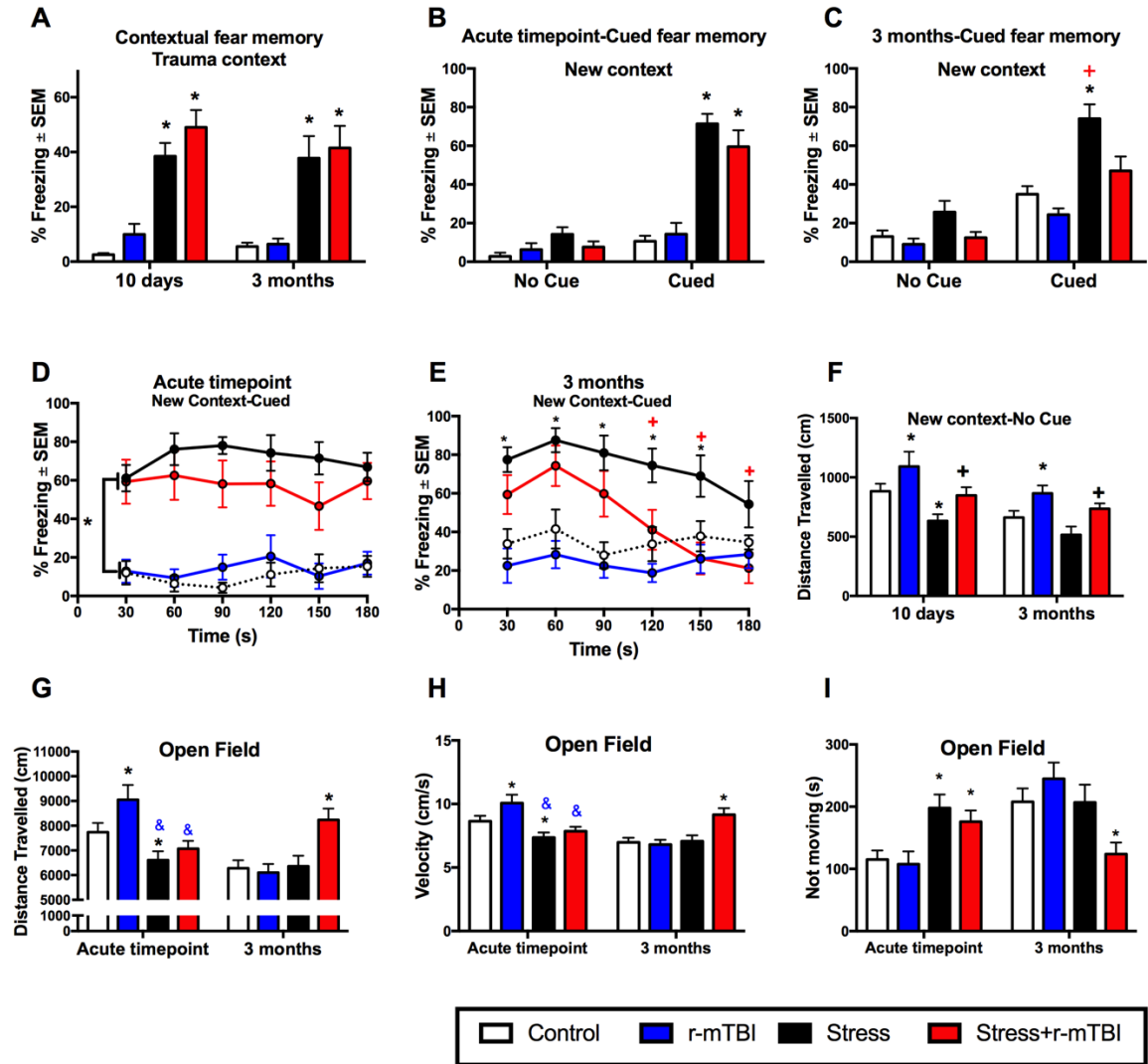


Figure 3.9. Effect of stress and r-mTBI on fear memories, anxiety and stress-coping behavior at the acute and 3-month timepoints. (A) Stress and stress+r-mTBI groups demonstrated similar increases in freezing time relative to controls at the acute and 3-month timepoints. r-mTBI didn't affect recall of cued fear memories in animals exposed to RUS at the acute timepoint, as stress and stress+r-mTBI animals showed similar freezing time relative to control animals (B) and (D). However, at the 3-month timepoint, stress+r-mTBI animals showed an attenuation in recall of cued fear memories compared to stress-only animals in the total test duration (3 min) as demonstrated by reduced freezing times relative to the stress-only group (C). The stress group showed increased freezing time relative to controls in the first 150 seconds of the test, while the stress+r-mTBI group showed an increased freezing time only during the first 60 seconds (E). The distance travelled (G) and velocity (H) in the open field were increased in the r-mTBI-only group and reduced in the stress and stress+r-mTBI groups relative to their respective control groups at the acute timepoint (A). At the chronic timepoint, animals in the stress+r-mTBI group showed distance travelled and velocity (B) and decreased not-moving time (I) relative to the control group. All data were analyzed using repeated measures Two-Way

ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons (n=10-11). Statistically significant discoveries versus the control group are denoted by “*”, while statistically significant discoveries versus the stress+r-mTBI group are denoted by “+”. Statistically significant discoveries versus the r-mTBI group are denoted by “&”.

The Open Field test has been widely used to evaluate overall locomotion and exploratory behavior in rodents (Hall and Ballachey 1932). The test can also be used to examine anxiety-like behavior as long as the total ambulatory distance covered by all study groups is the same (Seibenhener and Wooten 2015). At the acute timepoint, the overall locomotion activity was reduced after stress and increased by r-mTBI as demonstrated by decreased distance travelled in stress-only mice and increased distance travelled in the r-mTBI-only mice relative to control mice (Two-Way ANOVA: interaction $F(3, 94) = 8.6$, $p < 0.0001$, main stress/r-mTBI effect $F(3, 94) = 3.38$, $p = 0.024$ with correction for multiple comparisons, **Figure 3.9G**). Stress+r-mTBI mice showed a reduction in the total distance travelled in the Open Field relative to r-mTBI-only mice, but not relative to the control group, possibly due to opposing effects of stress and r-mTBI on locomotion at the acute timepoint. Interestingly, at the 3-month timepoint, stress+r-mTBI groups showed increased locomotion relative to controls, r-mTBI-only and stress-only groups (Two-Way ANOVA: interaction $F(3, 94) = 8.6$, $p < 0.0001$, main stress/r-mTBI effect $F(3, 94) = 3.38$, $p = 0.024$ with correction for multiple comparisons, **Figure 3.9G**). Examination of Open Field average velocity (**Figure 3.9H**) and inactivity time (**Figure 3.9I**) showed additional evidence of the altered ambulatory behavior after stress and/or r-mTBI. The distance travelled in the Open Field correlated negatively with the immobility time only in stress+r-mTBI group (Pearson correlation: $r = -0.71$, $p = 0.022$, **Figure 3.10D**). No changes in the

time spent in the Open Field center zone ($p>0.05$, **Figure 3.11A**) or center zone entries ($p>0.05$, **Figure 3.11B**) were observed at any timepoint.

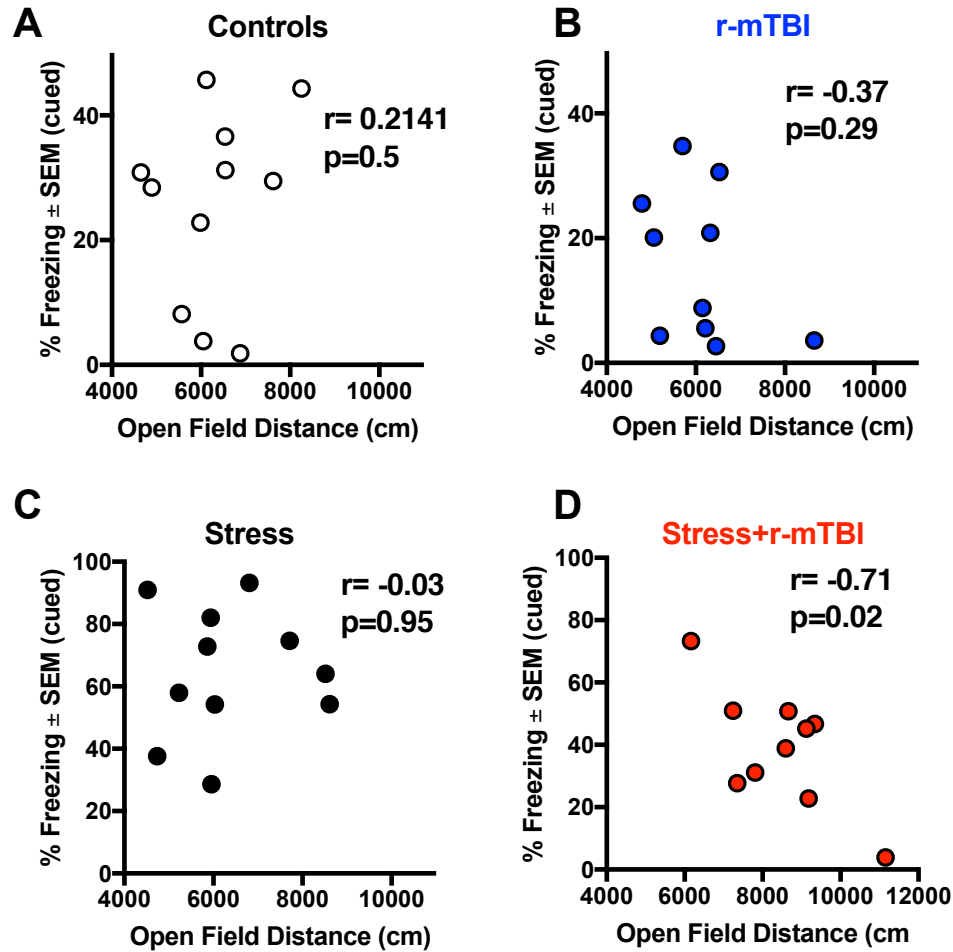


Figure 3.10. Correlations between the distance travelled in the open field and the % time freezing in the cued fear memory test in different study groups at 3 months after second RUS. The distance travelled by each animal in the open field correlated negatively with freezing scores in the cued fear memory test in the stress+r-mTBI group (D), but not in control (A), r-mTBI (B) or stress-only (C) groups. All data were analyzed using Pearson correlations.

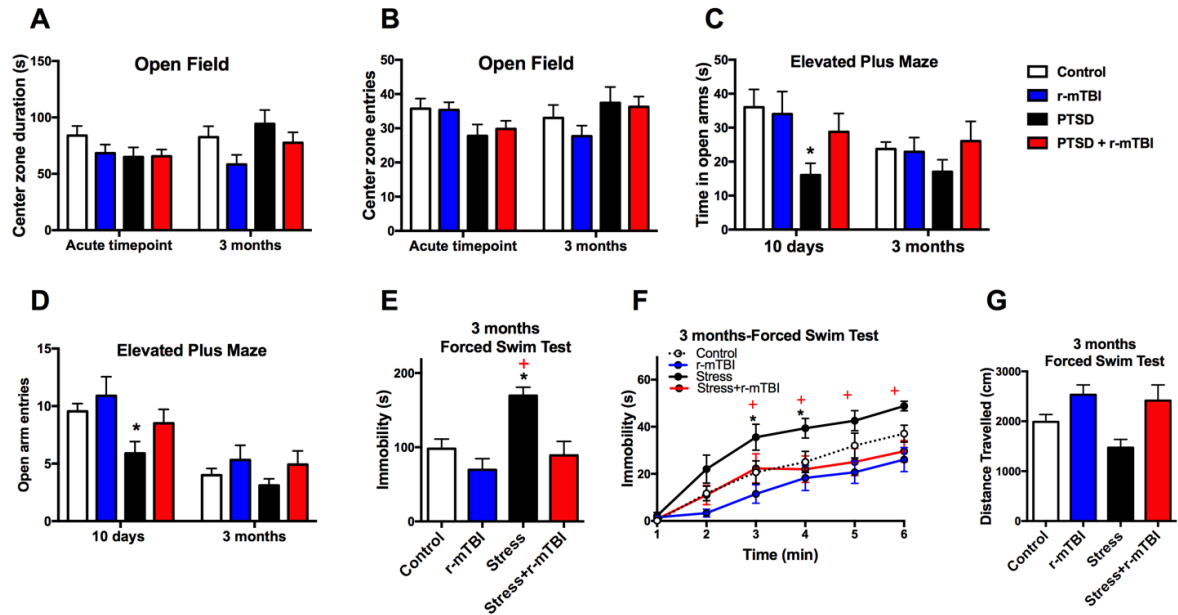


Figure 3.11. Effect of stress and r-mTBI on performance in the Elevated Plus Maze and Forced Swim Test. No changes in the Open Field center zone time (A) or entries (B) were observed at any timepoint. Stressed animals spent less time in the EPM open arms (C) and made less open arm entries (D) at the acute timepoint. Average immobility time in the last 4 min was increased in stress-only mice compared to the controls, r-mTBI and stress+r-mTBI groups (E) at 3 months after second RUS. Animals in the stress-only group displayed increased immobility time in minutes 3 to 6 of the FST when compared to the r-mTBI and stress+r-mTBI groups (F). There was a trend towards increased distance travelled in r-mTBI animals relative to controls in the FST (G). Data in A-D, F were analyzed using RM-Two-Way ANOVA ($n = 10-15$). Data in E, G were analyzed using a One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons ($n=10-11$). Statistically significant discoveries versus the control group are denoted by “*”, while statistically significant differences between the stress-only and stress+r-mTBI groups are denoted by “+”.

Experiment 2. Effect of r-mTBI on anxiety-like behavior and stress-coping strategies.

I examined the effect of r-mTBI on anxiety-like behavior and stress-coping strategies using the Elevated Plus Maze (EPM) and the Forced Swim Test (FST), respectively. Animals exposed to stress-only showed anxiety-like behavior at the acute timepoint as demonstrated by reduced time spent in the open arms (RM-Two-Way ANOVA: interaction $F(3, 34) = 1.137$, $p > 0.05$, main stress/r-mTBI effect $F(3, 34) = 2.968$,

$p=0.054$ with correction for multiple comparisons, **Figure 3.11C**) and arm entries in the EPM (RM-Two-Way ANOVA: interaction $F(3, 35) = 1.53$, $p>0.05$, main stress/r-mTBI effect $F(3, 35) = 2.96$, $p=0.045$ with correction for multiple comparisons, **Figure 3.11D**). No significant stress or r-mTBI effects were observed at the 3-month timepoint in the elevated plus maze ($p>0.05$, **Figure 3.11C** and **3.11D**). The validity of retesting at the 3-month timepoint is questionable since control animals showed a reduction in time spent in the open arms and number of entries into the open arms after retesting (**Figures 3.11C** and **3.11D**). At 3 months after the second RUS, animals in the stress-only group showed negative stress-coping behavior (increased immobility time relative to controls in the FST) (One-way ANOVA, $F(3, 37) = 8.024$, $p=0.0003$, with correction for multiple comparisons, **Figure 3.11E**) and (RM-Two-Way ANOVA: interaction $F(15, 185) = 1.55$, $p>0.05$, main stress/r-mTBI effect $F(15, 158) = 62.66$, $p=0.003$ with correction for multiple comparisons, **Figure 3.11F**). r-mTBI ameliorated the effects of RUS on stress-coping behavior as evidenced by decreased immobility time in the stress+r-mTBI group compared to the stress-only group (**Figure 3.11E**). The total distance travelled in the FST is depicted in **Figure 3.11G**. A trend towards increased total distance travelled was observed for r-mTBI relative to controls. Similar to our findings in the cued fear memory test, the increased locomotor activity after r-TBI may account for the reduced immobility time in the stress+r-mTBI group relative to stress-only group in the FST.

Stress modulates acute r-mTBI impairments in spatial learning and memory.

Our r-mTBI mouse model is associated with persistent and progressive deficits in spatial memory and learning, evaluation of which has previously utilized the Barnes Maze

(Mouzon et al. 2014). The performance of each group during training sessions in a radial arm water maze (RAWM) is depicted in **Figures 3.12A**. Mice received two sets of acquisition training sessions (5 days each). The first set started at the acute timepoint followed by a second set 3 months after. All groups demonstrated a learning curve during the two sets of training (Linear regression, slopes >0 , $P<0.0001$, **Figures 3.12A**). The mean number of memory errors at training day 5 was used to evaluate spatial memory at the acute timepoint (**Figures 3.12B**), while the mean number of memory errors at training day 10 was used to assess spatial memory at the 3-month timepoint (**Figures 3.12D**). In addition, the first day of the second training session (Day 6) was used to evaluate the long-term recall of spatial memory (**Figures 3.12C**). At the acute timepoint, only the r-mTBI group showed increased mean memory errors relative to the control group (One-way ANOVA, $F(3, 34) = 5.75$, $p = 0.003$, with correction for multiple comparisons, **Figures 3.12A**), while at the chronic timepoint both r-mTBI-only and stress+r-mTBI groups showed increased mean memory errors when compared to control mice (One-way ANOVA, $F(3, 34) = 7.23$, $p = 0.0007$, with correction for multiple comparisons, **Figures 3.12D**). Mice in stress, r-mTBI and stress+r-mTBI groups made more memory errors during the 3-month recall when compared to the control group (One-way ANOVA, $F(3, 34) = 3.76$, $p = 0.0031$, with correction for multiple comparisons, **Figures 3.12C**).

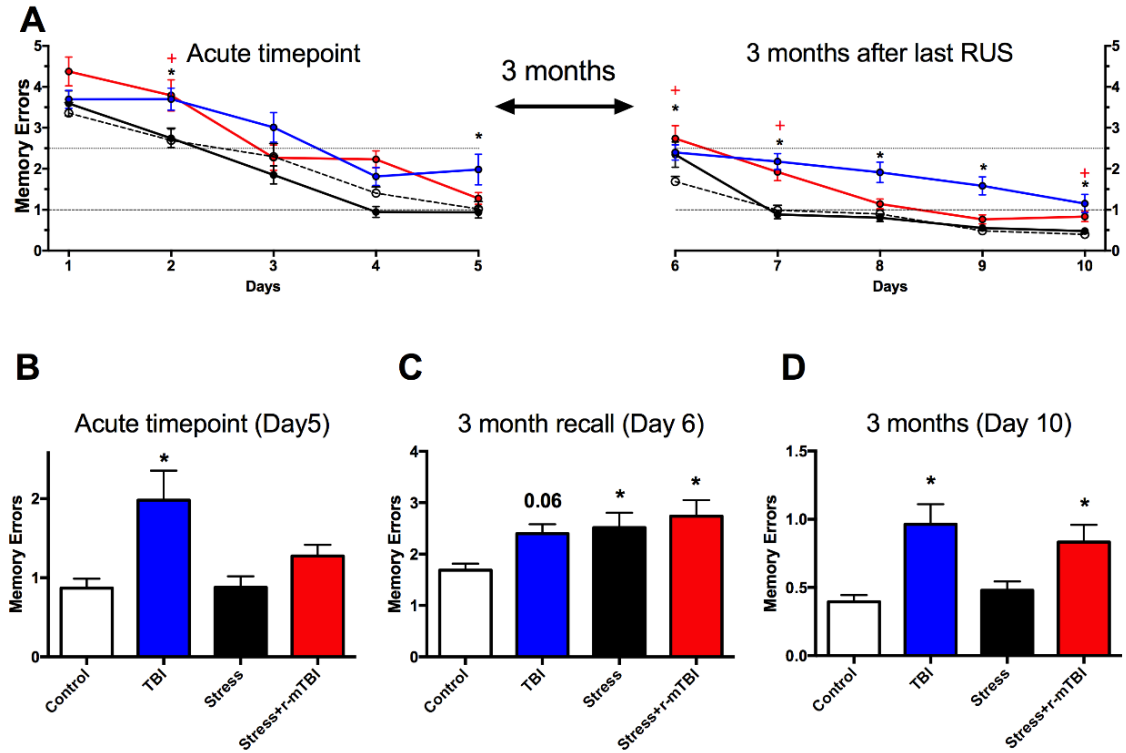


Figure 3.12. Effect of stress and r-mTBI on spatial learning and memory. The performance of each group during two separate 5-day training sessions in a radial arm water maze (RAWM) is depicted in panel (A). In the last trial at the acute timepoint (Day5), only animals in the r-mTBI group showed spatial memory deficits as evidenced by a significant increase in the mean memory errors relative to controls (B). All treatment groups showed increased mean memory errors relative to controls in the 3-month recall of learned memories (Day6) (C). A main r-mTBI effect was observed in the last trial at the 3-month timepoint (Day10) as animals in the r-mTBI and stress+r-mTBI groups showed increased mean memory errors relative to controls (B). Data in A were analyzed using repeated measures Two-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons (n=10-11), while data in C-D were analyzed using a One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons (n=10-11). In panel A, statistically significant discoveries for the r-mTBI group versus the control group are denoted by “*”, while statistically significant discoveries for the stress+r-mTBI group versus the control group are denoted by “+”. In panels B-D, statistically significant discoveries versus the control group are denoted by “*.”

The 3-chamber test was used to assess social interaction and social memory after RUS and r-mTBI. At the acute timepoint, as expected, control mice showed increased preference for the stranger mouse over the empty cage (One sample t-test: $t_{(10)}=3.577$, $p=0.005$, **Figure 3.13A**), while mice receiving stress-only, r-mTBI-only, and stress+r-mTBI showed no preference for the stranger mouse over the empty cage (One sample t-test: $p>0.05$, **Figure 3.13A**). All the groups showed no preference for the stranger mouse over the empty cage, when retested for social interactions at the 3-month timepoint (One sample t-test: $p>0.05$, **Figure 3.13A**). In the subsequent social memory test, no group differences were observed at the acute timepoint (One sample t-test: $p>0.05$, **Figure 3.13B**). However, at the 3-month timepoint, control animals and stress-only animals showed a preference for stranger 2 over stranger 1 (One sample t-test: $t_{(9)}=6.5$, $p=0.0001$ for controls, $t_{(9)}=3.2$ $p=0.001$ for the stress-only group, **Figure 3.13B**), while r-mTBI and stress+r-mTBI groups spent equal time with both strangers ($p>0.05$, **Figure 3.13B**). The lack of preference for stranger 2 over stranger 1 in the social memory test suggests that social memory was impaired in r-mTBI and stress+r-mTBI animals (One sample t-test: $p>0.05$, **Figure 3.13B**).

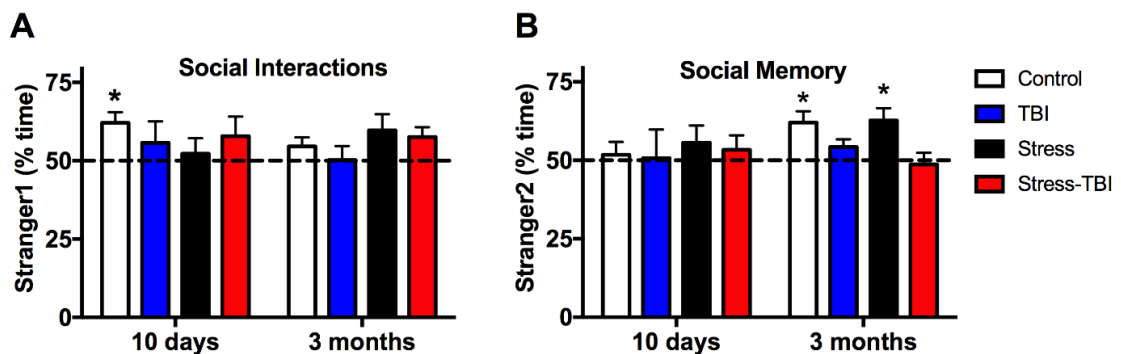


Figure 3.13. Effect of stress and r-mTBI on social interactions and social memory at the acute timepoint and at 3 months after the second RUS. In the social interaction test, only the control group showed increased preference for stranger1 relative to the empty cage at the acute timepoint (A). All groups showed no preference for stranger1 when retested at 3 months after the second RUS (A). In the social memory test, all groups failed to show a preference for stranger2 at the acute timepoint (B). However, at the 3-month timepoint, control and stress-only groups showed increased preference for stranger2 over stranger1. In (A), the ratio between time spent around stranger1 cage to total time spent around stranger1 and the empty cage was calculated for each animal to evaluate social interactions, while in (B), the ratio between time spent around stranger2 cage (new never seen mouse) to the total time around both strangers was calculated to evaluate social memory (social novelty recognition). Data in A and B were analyzed using One sample t test.

Experiment 2. No dysregulation in baseline HPA-axis markers after repeated exposure to RUS.

A single exposure to the RUS paradigm is associated with chronic HPA axis dysregulation (low baseline plasma corticosterone levels in stressed animals) as per our previous model characterization (Algamal et al. 2018). In order to understand the effects of repeated RUS exposure on the HPA-axis, I first measured baseline plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels at 3 months after second RUS. Surprisingly, no significant changes were observed in baseline plasma ACTH or corticosterone levels after repeated exposure to RUS at the chronic timepoint ($p > 0.05$, $p > 0.05$, **Figure 3.14A and 3.14B**). In addition, baseline ACTH and corticosterone levels did not change after r-mTBI ($p > 0.05$, **Figure 3.14A and 3.14B**). Similarly, baseline plasma cortisol, norepinephrine and neuropeptide Y levels did not change after stress and/or r-mTBI ($p > 0.05$, **Figure 3.14C-E**). No changes in the levels of corticosterone, cortisol, norepinephrine and NPY were observed in any group at the acute timepoint ($p > 0.0$, **Figure 3.15**). I then examined the regulation of glucocorticoids in the brain by measuring the expression levels of glucocorticoid receptor (GR), FK506 binding protein

51 (FKBP51) and corticotropin-releasing hormone (CRH) in the hypothalamus (**Figure 3.14**). Levels of FKBP51 and CRH were downregulated in the hypothalamus of r-mTBI and stress+r-mTBI animals (t-test: $t_{(9)}=2.4$, $p=0.042$, **Figure 3.14G and 3.14H**) at 3 months after the second RUS. Exposure to RUS did not affect the expression of GR, FKBP51, or CRH in the hypothalamus (**Figure 3.14F-H**).

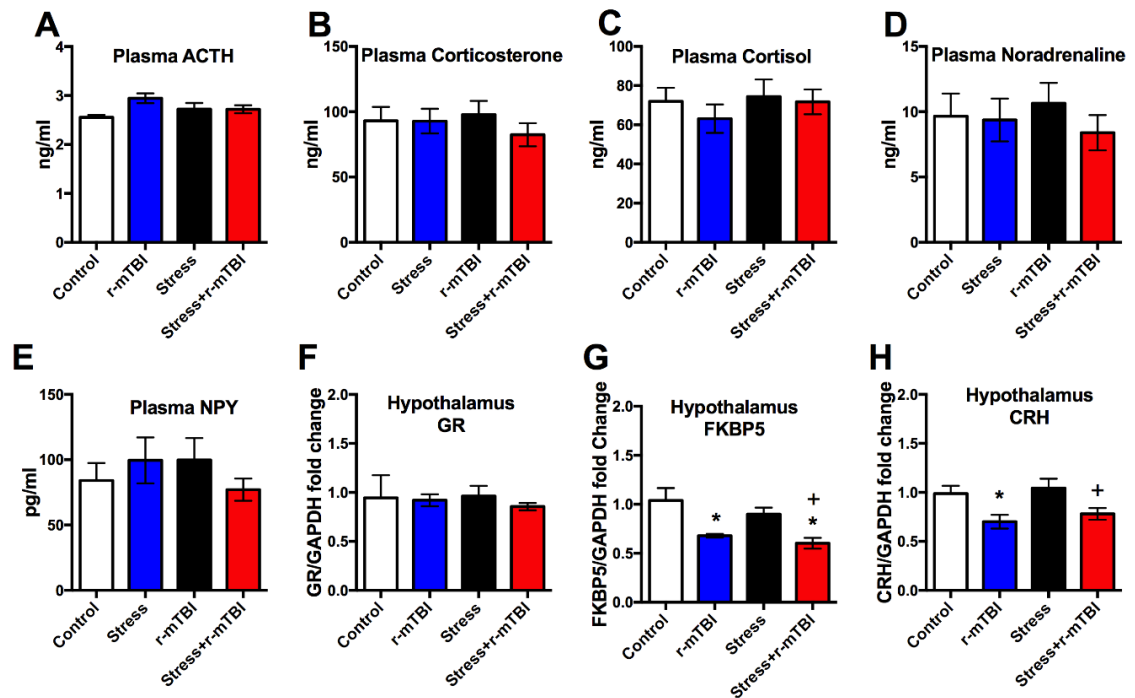


Figure 3.14. Effect of stress and r-mTBI on HPA axis markers at 3 months after the second RUS. No changes in plasma levels of ACTH (A), corticosterone (B), cortisol (C), noradrenaline (D) or neuropeptide Y (NPY) (E) were detected at 3 months after the second RUS. Quantification of western blot images for GR (F), FKBP51 (G), and CRH (H) levels in the hypothalamus at 3 months after the second RUS. Levels of FKBP51 and CRH were reduced in r-mTBI and stress+r-mTBI groups relative to the control and stress-only groups, respectively. Data A-F were analyzed using a One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons ($n=4-6$). Statistically significant discoveries versus the control group are denoted by “*”, while statistically significant discoveries for the stress-only group are denoted by “+”.

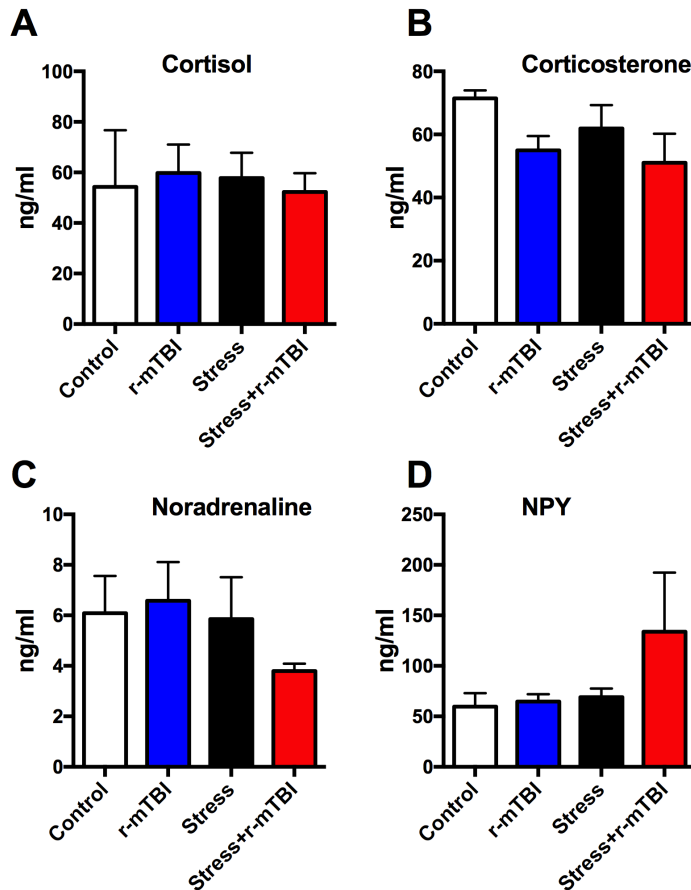


Figure 3.15. Profiling of plasma stress markers at the acute timepoint. No changes in plasma levels of cortisol (A), corticosterone (B), noradrenaline (C) and neuropeptide Y (D) were observed at the acute timepoint. Data in A-D were analyzed using One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons. 3-5 plasma samples per group were used at the acute timepoint.

Experiment 2. Effect of stress and r-mTBI on the immune system.

To examine the effect of repeated RUS and r-mTBI on the peripheral immune system, I measured plasma cytokine levels at baseline and after ex-vivo stimulation of whole blood with lipopolysaccharide (LPS) at the chronic timepoint. At baseline, IL2 levels were reduced in the stress groups (i.e. stress and stress+r-mTBI groups) relative to the control group (One-way ANOVA, $F(3, 30) = 3.7$, $p = 0.023$, with correction for multiple

comparisons, Table 3.1), while IL5 levels were reduced in r-mTBI groups (i.e. r-mTBI and stress+r-mTBI groups) relative to the control group (One-way ANOVA, $F(3, 30) = 3.1$, $p = 0.041$, with correction for multiple comparisons, **Table 3.1**). Additionally, baseline plasma IL10 levels were reduced in all stress and/or r-mTBI groups (One-way ANOVA, $F(3, 30) = 4.3$, $p = 0.012$, with correction for multiple comparisons, **Table 3.1**) relative to the control group. Baseline levels of other cytokines were not significantly altered (**Table 3.1**). In the ex-vivo whole blood challenge, IL1 β , IL10, TNF α , IL6, and IL12 levels were increased in all treatment groups after incubation with LPS for 6 hours (RM-Two-Way ANOVA: interaction $p > 0.05$, main LPS effect, $p < 0.0013$, stress and/or r-mTBI effect, $p > 0.05$ **Figure 3.16E-I**). Although the levels of TNF α showed an elevated trend in the stress-only animals relative to control animals after LPS stimulation (**Figure 3.16I**), RM-Two-way ANOVA analysis showed no main stress and/or r-mTBI effect.

Table 3.1. Effect of stress and/or r-mTBI on cytokines levels in brain and plasma

Baseline cytokine levels (mean \pm SEM) at 3 months after the second RUS										
Plasma (pg/mL)					p	Hippocampus (pg/mg protein)				p
	Contro l	r- mTBI	Stress	Stress + r- mTBI		Contro l	r- mTBI	Stress	Stress + r- mTBI	
IFγ	1.85 \pm 0.30	1.58 \pm 0.14	1.30 \pm 0.12	1.24 \pm 0.09	0.08	1.91 \pm 0.9	1.18 \pm 0.23	1.554 \pm 0.77	2.75 \pm 1.37	0.68
TNFα	8.74 \pm 0.82	7.52 \pm 0.59	8.28 \pm 1.57	6.4 \pm 0.76	0.38	0.049 \pm 0.09	0.06 \pm 0.09	0.052 \pm 0.07	0.053 \pm 0.056	0.61
IL1β	0.101 \pm 0.03	0.081 \pm 0.01	0.079 \pm 0.02	0.072 \pm 0.01	0.44	24.68 \pm 3.57	19.71 \pm 4.05	18.64 \pm 1.878	21.65 \pm 3.12	0.61
IL2*	0.505 \pm 0.09	0.387 \pm 0.05	0.272 \pm 0.06	0.248 \pm 0.05	0.02	43.73 \pm 12.2	21.29 \pm 10.61	25.15 \pm 5.98	31.2 \pm 4.9	0.41
IL4	0.015 \pm 0.008	0.026 \pm 0.008	0.022 \pm 0.01	0.04 \pm 0.01	0.27	13.56 \pm 1.93	15.95 \pm 4.13	16.91 \pm 1.8	12.92 \pm 2.09	0.63
IL5*	0.664 \pm 0.04	0.473 \pm 0.07	0.609 \pm 0.08	0.507 \pm 0.03	0.05	19.03 \pm 2.27	19.39 \pm 2.74	22.58 \pm 1.02	20.47 \pm 1.382	0.68
IL6	14.65 \pm 4.3	25.28 \pm 7.9	11.42 \pm 1.8	8.05 \pm 1.3	0.30	1.585 \pm 0.35	1.191 \pm 0.14	1.423 \pm 0.27	1.04 \pm 0.047	0.33
IL10*	16.12 \pm 2.3	11.08 \pm 1.1	10.56 \pm 0.65	10.8 \pm 0.68	0.01	281.3 \pm 32.05	346.8 \pm 36.25	292.6 \pm 27.08	294 \pm 17.16	0.45
IL12	2.9 \pm 1.7	2.7 \pm 2.0	7.6 \pm 4.5	4.2 \pm 1.9	0.71	0.57 \pm 0.33	0.155 \pm 0.155	0.09 \pm 0.09	0.71 \pm 0.29	0.30

Asterisks denote statistical significance (*p < 0.05).

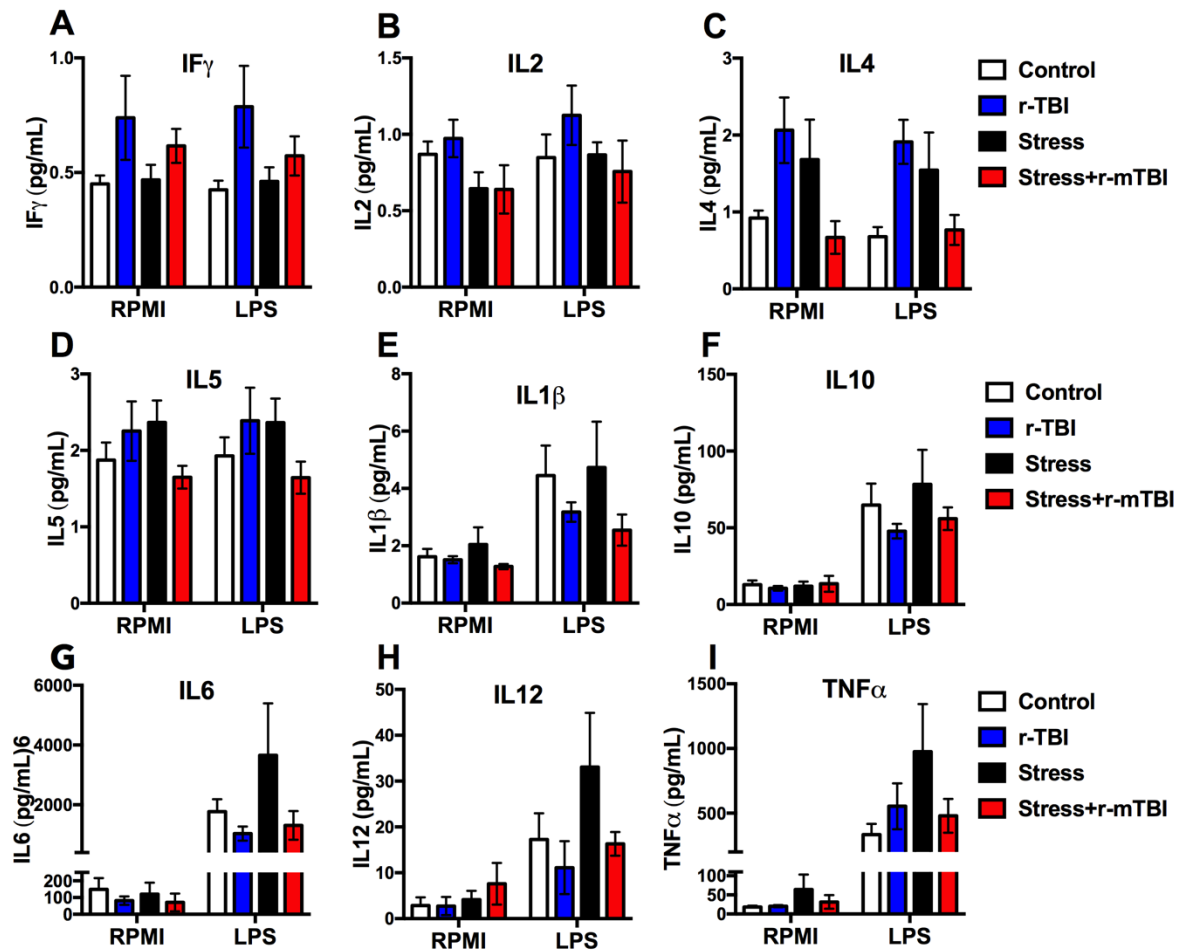


Figure 3.16. Effect of stress and r-mTBI on LPS-induced cytokine production in whole blood. Plasma levels of IF γ (A), IL2 (B), IL4 (C), and IL5 (D) were not altered after incubating whole blood with LPS for 6 hours. On the contrary, the production levels of IL1 β , IL10, IL6, IL12, and TNF α were significantly increased 6 hours after LPS stimulation. No stress or r-mTBI effects were observed in any group after LPS stimulation. Data in A-I were analyzed using repeated measures Two-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons (n=5-6).

Experiment 2. Stress augments r-mTBI-dependent microgliosis and astrogliosis in the corpus callosum

To assess the effect of stress on inflammatory markers associated with repetitive mTBI, brain tissue from different treatment groups was immunostained for GFAP and Iba1

at 3 months following the second RUS. In the corpus callosum, r-mTBI was associated with increased GFAP levels relative to control animals, while stress+r-mTBI animals showed increased GFAP levels relative to r-mTBI-only and control animals (Two-Way ANOVA: interaction $F(6, 36) = 7.92$, $p < 0.0001$, with correction for multiple comparisons, **Figure 3.17A**). Similarly, Iba1 levels were increased in the corpus callosum of stress+r-mTBI mice relative to r-mTBI-only mice, with both groups showing significantly higher levels than controls (Two-Way ANOVA: interaction $F(6, 36) = 9.126$, $p < 0.0001$, with correction for multiple comparisons, **Figure 3.17B**). No significant changes in GFAP or Iba1 immunostaining were observed in the cortex or the hippocampus after stress or r-mTBI (**Figure 3.17A and 3.17B**). Representative images for GFAP and Iba1 staining are depicted in **Figure 3.18**. I also quantified the levels of neurofilament heavy chains (NF-H) and medium chains (NF-M) to explore the impact of r-mTBI on axonal injury. While there was no change in the hippocampal levels of NF-H (**Figure 3.17C**, $P > 0.05$), NF-M levels were elevated in the r-mTBI group, but not in the stress+r-mTBI group (One-way ANOVA, $F(3, 17) = 5.5$, $p = 0.0081$, with correction for multiple comparisons, **Figure 3.17D**). Rep

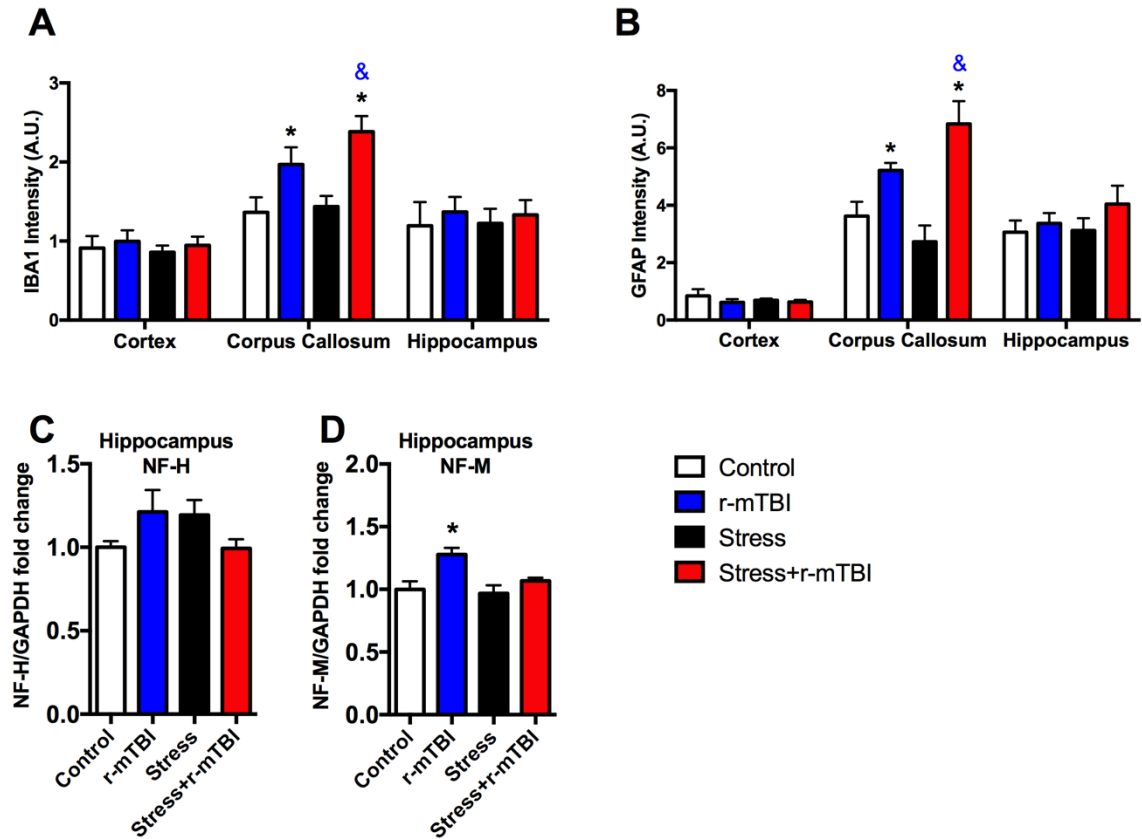


Figure 3.17. Effect of stress on brain injury markers at 3 months after last injury.

Quantitative assessments of Iba1 and GFAP immunostaining in different brain regions are depicted in panels A and B, respectively. Levels of Iba1 (A) and GFAP (B) were upregulated at 3 months after the last injury in the corpus callosum of r-mTBI and stress+r-mTBI animals. Stress increases microgliosis and astrogliosis in injured animals as demonstrated by increased levels of Iba1 (A) and GFAP (B) immunostaining in the stress+r-mTBI group relative to the r-mTBI-only group. No changes in GFAP or Iba1 were observed in the cortex or the hippocampus (A, B). Quantification of hippocampal neurofilament heavy chain (C), neurofilament medium chain (D), at 3 months after the second RUS using western blotting. Levels of hippocampal neurofilament medium chain (D) were increased in r-mTBI-only group relative to controls. Data in A-B were analyzed using Two-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons (n=5-6). Data C-D were analyzed using a One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons (n=4-6). Statistically significant discoveries versus the control group are denoted by “*”, while statistically significant discoveries versus the r-mTBI group are denoted by “&”.

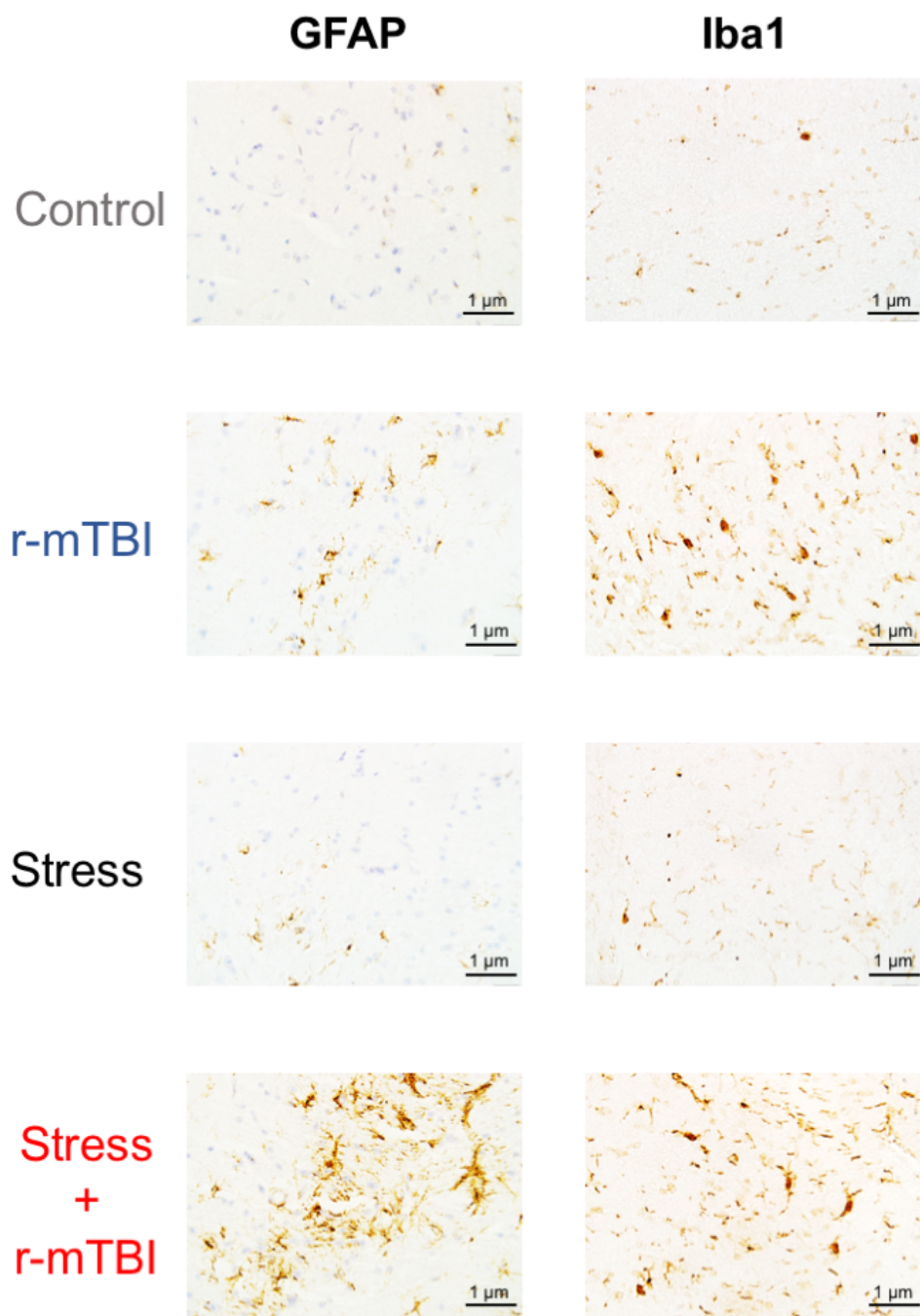


Figure 3.18. Representative GFAP and Iba1 immunostaining images form each study group.

Experiment 2. Reduced spine density and chronic alterations in synaptic plasticity markers after r-mTBI and/or stress exposures.

Variations in dendritic spine density and morphology have been reported after stress. To examine the chronic effects of repeated RUS and r-mTBI on spine density, I used the Golgi-cox staining method to quantify the number of spines per 10 μm dendritic length in the prefrontal cortex and in the hippocampus at 3 months after the second RUS. **Figure 3.19A** shows representative images from the Golgi-stained hippocampal CA1 neurons. Stress and/or r-mTBI did not change spine density in the prefrontal cortex (One-way ANOVA, $P > 0.05$, **Figure 3.19B**). However, stress and/or r-mTBI resulted in a reduction in spine density in hippocampal CA1 pyramidal neurons (One-way ANOVA, $F(3, 14) = 4.45$, $p = 0.0215$, with correction for multiple comparisons, **Figure 3.19C**). I then examined the expression levels of notable markers/mediators of neuroplasticity using immunoblot analysis of hippocampal lysates. GluN2A/GluN2B ratio and the levels of CAMKII α were downregulated with stress and/or r-mTBI relative to controls (One-way ANOVA, $F(3, 16) = 5.4$, $p = 0.009$, with correction for multiple comparisons, **Figure 3.19H**, respectively) and (One-way ANOVA, $F(3, 16) = 6.62$, $p = 0.004$, with correction for multiple comparisons, **Figure 3.19I**, respectively). GluNR1 levels were downregulated in all groups relative to controls at the acute timepoint (One-way ANOVA, $F(3, 13) = 6.01$, $p = 0.0008$, with correction for multiple comparisons, **Figure 3.20A**) and upregulated in stress+r-mTBI groups relative to controls at the 3-months timepoint (One-way ANOVA, $F(3, 16) = 4.04$, $p = 0.0258$, with correction for multiple comparisons, **Figure 3.19G**) at the 3-month timepoint. Levels of PSD-95 showed a trend towards reduction only in the stress group (One-way ANOVA, $F(3, 16) = 4$, $p = 0.004$, with correction for multiple comparisons, **Figure 3.19J**) at the 3-month timepoint. The levels of other neuroplasticity markers such

as GluN2B, P-JNK/JNK and P-AKT/AKT were not significantly altered after RUS and r-mTBI ($P > 0.05$, Figure 3.19 and 3.20).

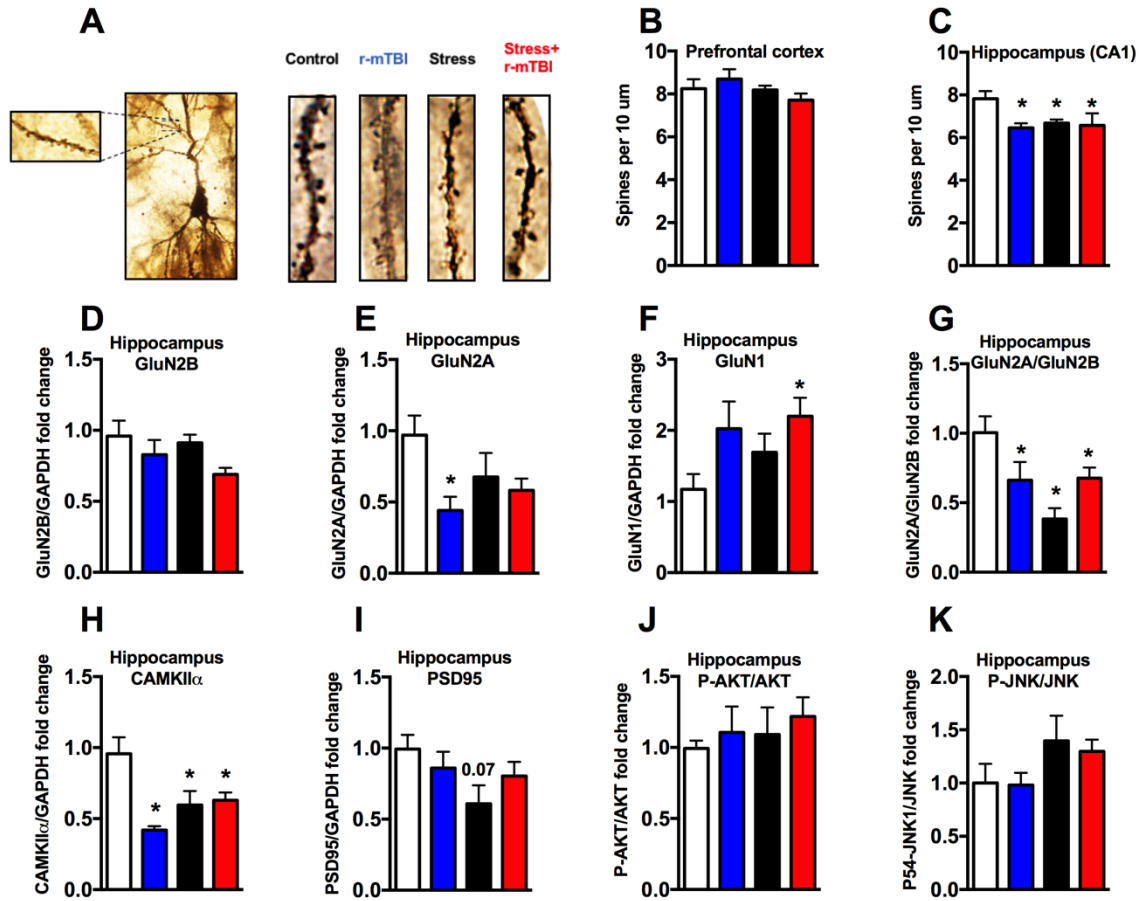


Figure 3.19. Effect of stress and r-mTBI on spine density and a number of synaptic markers at 3 months after the second RUS. Representative dendritic segments of the hippocampal CA1 region (A). Quantification of spine density in the PFC (B) and CA1 of the hippocampus (C). Stress, stress+r-mTBI and r-mTBI groups showed reduced spine density only in the hippocampal CA1 region when compared to the control group. Quantification of Western Blot images for GluN2B (D), GluN2A (E), GluN1 (F), GluN2A/GluN2B ratio (G), CAMKII α (H), PSD-95 (I), the ratio of p-AKT/AKT (J), and P-JNK/JNK (K) in the hippocampus at 3 months after the second RUS. Data in B-L were analyzed using a One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons ($n=4-6$). Statistically significant discoveries versus the control group are denoted by “*”.

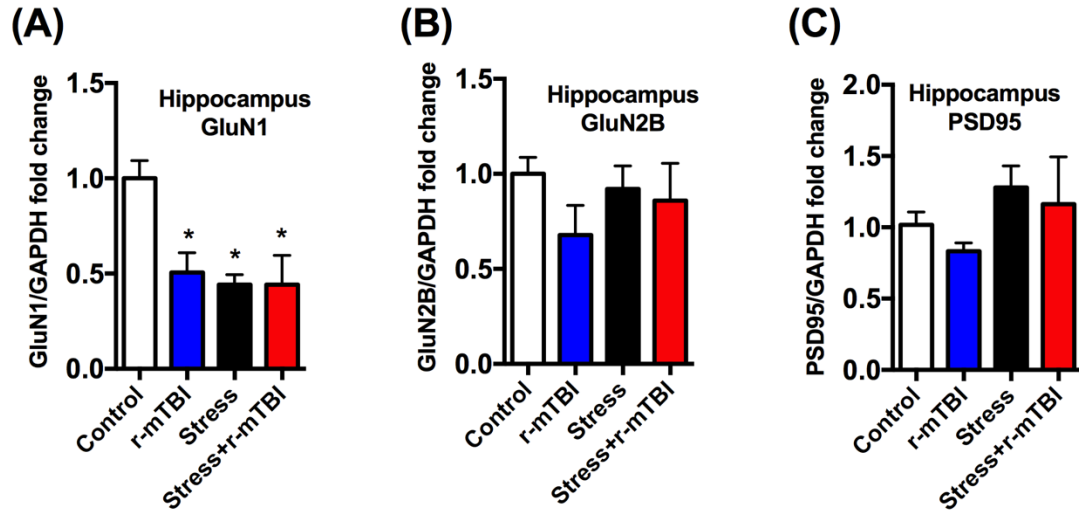


Figure 3.20. Effect of stress and r-mTBI on a number of hippocampal synaptic plasticity markers at the acute timepoint. Quantification of GluN1 (A), GluN2B (B), and PSD-95 (C) levels in the hippocampus at the acute timepoint. Levels of GluN1 were significantly reduced in the stress, r-mTBI and stress+r-mTBI groups compared to the control group. Data A-C were analyzed using a One-Way ANOVA followed by two-stage linear step-up procedure of BKJ to correct for multiple comparisons (n=4-5). Statistically significant discoveries versus the control group are denoted by “*”.

Experiment 2. Effect of stress and r-mTBI on neurotrophic signaling markers.

BDNF levels in the hippocampus were not altered at 3 months after exposure to RUS and r-mTBI ($p > 0.05$, **Figure 3.21A**), while BDNF precursor (ProBDNF) levels were only increased in the r-mTBI group (One-way ANOVA, $F(3, 34) = 7.23$, $p = 0.0007$, with correction for multiple comparisons, **Figure 3.21C**). I did not find any detectable changes in levels of BDNF receptor (TrkB) or proBDNF receptor (P75NTR) in the hippocampus at 3 months after the second RUS (One-way ANOVA, $p > 0.05$, **Figure 3.21B** and **3.21D**). Interestingly, there was an effect for the combination of stress and r-mTBI on hippocampal P75NTR expression levels at the acute timepoint (One-way ANOVA, $F(3, 34) = 7.23$, $p = 0.0007$, with correction for multiple comparisons, **Figure 3.22D**). No changes in

BDNF, proBDNF or TrkB levels were detected at the acute timepoint (One-way ANOVA, $p>0.05$, **Figure 3.22**).

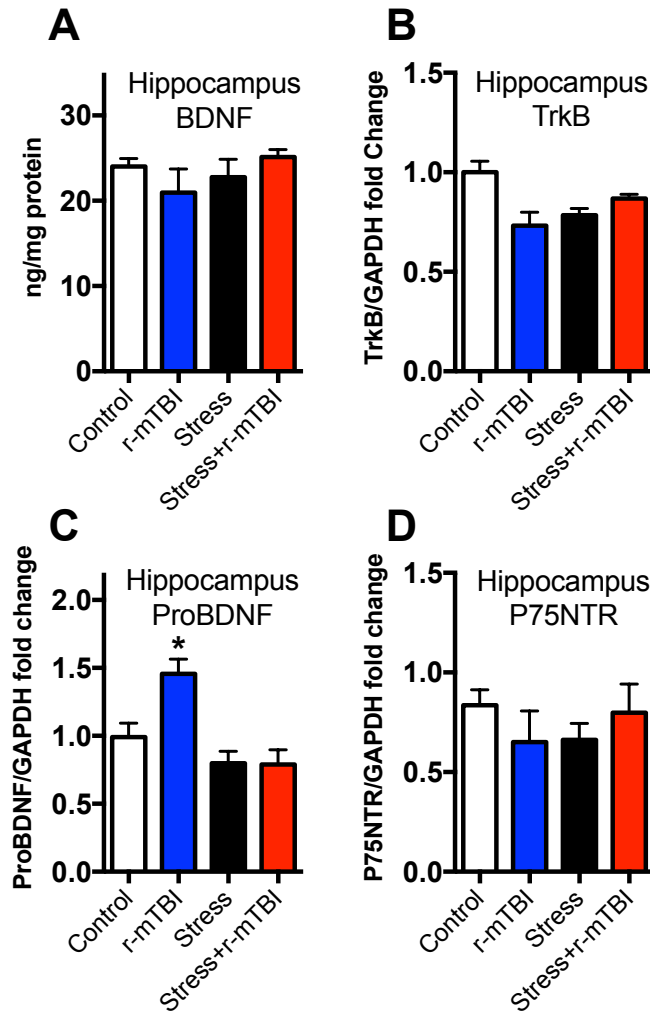


Figure 3.21. Effect of RUS on hippocampal neurotrophic factors and receptors at 3 months after the second RUS. Quantification of hippocampal TrkB (A), P75NTR (C), and ProBDNF (D) levels using western blotting. Levels of hippocampal BDNF were quantified using ELISA (B). Hippocampal ProBDNF levels were upregulated only in the r-mTBI group at 3 months after the second RUS. No changes in TrkB, BDNF or P75NTR were observed at the 3-month timepoint. Data in A-D were analyzed using One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons ($n=4-6$). Statistically significant discoveries versus the control group are denoted by “*”.

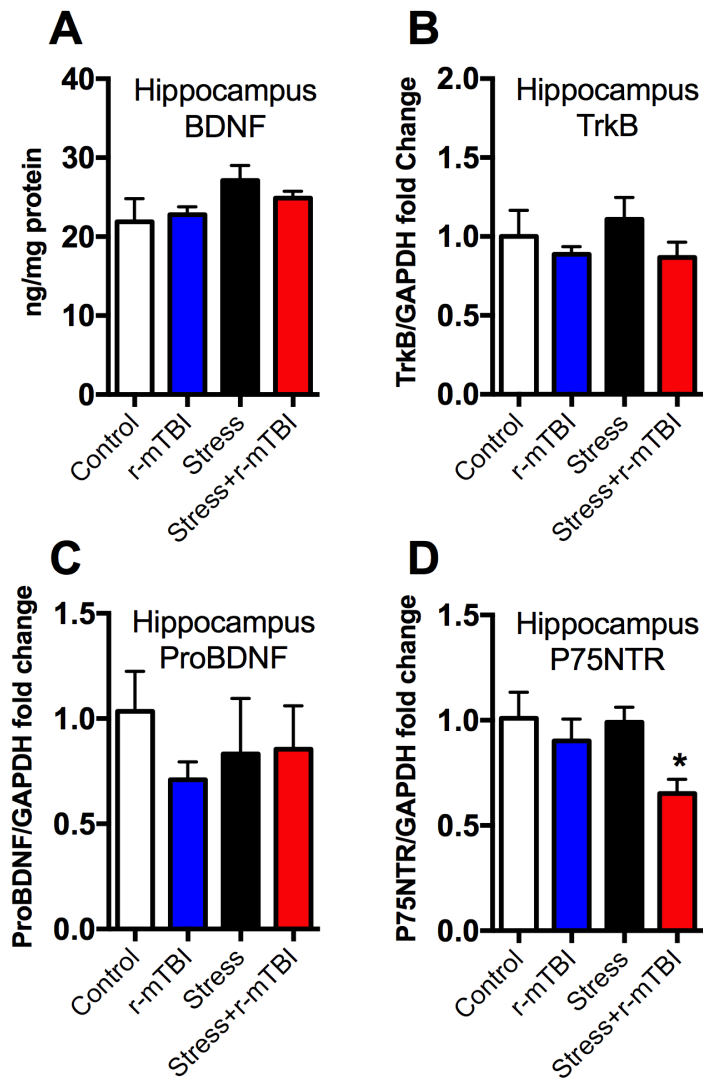


Figure 3.22. Effect of RUS on hippocampal neurotrophic factors and receptors at the acute timepoint. Levels of hippocampal BDNF were quantified using ELISA (A). Quantification of hippocampal TrkB (B), ProBDNF (C) and P75NTR (D) levels using western blotting. P75NTR levels were downregulated in the stress+r-mTBI group at the acute timepoint (C). No changes in TrkB, BDNF, or ProBDNF were observed at the acute timepoint. Data in A-D were analyzed using One-Way ANOVA followed by two-stage linear step-up procedure of BKY to correct for multiple comparisons (n=4-6). Statistically significant discoveries versus the control group are denoted by “*”.

3. 4 Discussion.

At the outset of this study, my goal was to first explore the behavioural and biological outcomes of exposure to repetitive mTBI and our RUS paradigm. From previous work, I explored this relationship by combining our 5 hit mTBI model to our 21-day RUS paradigm and demonstrated moderate behavioral effects of mTBI on stress related behavioral phenotypes. While this r-mTBI model reliably produces robust long-lasting spatial memory deficits in injured animals (Mouzon et al. 2012, 2014), a single exposure to the RUS paradigm was associated with moderate changes in stress-coping behavior and recall of cued fear memories at the chronic timepoint (Algamil et al. 2018). Thus, in this chapter, I extended this work, by focusing on the role of exposures to ‘multiple episodes’ of the same stressful event, as experienced by combat soldiers who undergo multiple deployments, or civilians exposed to multiple episodes of the same traumatic event (e.g. domestic violence, torture, or first-time responders).

To that end, I administered our 21 day-RUS paradigm on two occasions (separated by a few months) to intensify the stress procedures and combined this with two separate exposures to our 5hit mTBI paradigm, respectively.

In this chapter, I presented a detailed characterization of the long-term consequences of recurrent and combined exposure to our well-characterized models of repeated unpredictable stress (RUS) (Algamil et al. 2018) and mTBI (Mouzon et al. 2012, 2014). Repeated stress or mTBI alone caused unique and distinct behavioral and neurobiological alterations long after the last exposure to mTBI or RUS. Combining both exposures resulted in complex domain-dependent outcomes with no overall additive or opposite effects. Animals receiving repeated RUS alone showed several PTSD-like

behaviors such as acute weight loss, acute anxiety, long-term recall of contextual and cued fear memories, and chronic passive stress-coping behavior. Moreover, repeated RUS alone was associated with remodeling of dendritic spines and changes in several synaptic plasticity signaling markers in the hippocampus 3 months after the second RUS. The unique r-mTBI traits were typified by persistent spatial and social memory deficits, microgliosis, astrogliosis and axonal damage. Combined exposure to repeated r-mTBI and RUS resulted in an apparent amelioration of stress-related behaviors in the cued fear memory and forced swim tests at the 3-months timepoint. However, these observations should be interpreted carefully due to the confounding effects of variable locomotion between study groups. Animals in the comorbid group showed additional TBI-related deficits represented by hyperactivity and, spatial and social memory deficits at the chronic timepoint. Additionally, stress+r-mTBI mice had synergistic effects on injury dependent astrogliosis and microgliosis in the corpus callosum and mitigated the mTBI-dependent increase in pro-BDNF in the hippocampus.

Previously I showed that animals exposed to a single episode of our 21-day RUS paradigm around 3 months of age, demonstrate recall of cued fear memories, passive stress-coping behavior, low baseline plasma corticosterone and ACTH levels and reduced hippocampal BDNF levels at 6 months after the last exposure. Here, I hypothesized that exposure to the 21-day RUS paradigm at 3 months of age with re-exposure at 6 months of age would augment stress-related behavioral and neurobiological phenotypes examined at a similar post-exposure timepoint from our previous investigation (i.e. 3 months after the last-exposure). At the behavioral level, animals re-exposed to RUS showed the same phenotypes reported after a single exposure. However, I also observed strong recall of contextual fear memories 3 months after the second RUS, which was not observed in the

previous single exposure paradigm (Algamil et al. 2018). In contrast to my initial hypothesis, I found that re-exposure to RUS resulted in a reduction in the key neurobiological markers observed after a single RUS exposure. Stress-only animals showed no change in baseline plasma corticosterone and ACTH levels (**Figure 3.19**) and no change in hippocampal BDNF levels relative to control animals (**Figure 3.21**) at 3 months after the second RUS. The noticeable change in stress-coping strategies in the forced swim test suggests that HPA axis functionality is further altered after re-exposure to RUS. Additional experiments measuring corticosterone levels immediately after stress or after suppression with dexamethasone are required to better understand the complexity of HPA axis involvement in the current model.

A number of clinical and preclinical studies have suggested that mTBI is implicated in the development of PTSD or PTSD-like behavior (Bryant et al. 2009; Elder et al. 2012; Kennedy 2007). A study conducted on civilians showed that patients who survived a mild TBI were more likely to develop PTSD than patients with no brain injury (Bryant et al. 2009). Similar results were reported from a study within the military population, where mTBI was linked to increased self-reported post-traumatic symptoms (Kennedy et al. 2010). In animals, exposure to different mTBI types, such as blast (Elder et al. 2012), weight drop (Meyer et al. 2012) or fluid percussion (Reger et al. 2012) injuries, was associated with increased fear memory recall and anxiety-like behavior multiple days after the injury. In all of these studies, the injury was administered before fear conditioning and in the absence of any additional stress procedures (Elder et al. 2012; Meyer et al. 2012; Reger et al. 2012).

Studies examining symptom severity in co-morbid PTSD and TBI patients relative to PTSD alone have shown mixed findings. Patients with co-morbid PTSD and mild to

severe TBI had fewer intrusive memories than patients with PTSD alone (Hibbard et al. 1998). On the contrary, Simonović et al., reported more detachment and hyperarousal symptoms in patients with co-morbid PTSD and mild TBI compared to those with PTSD alone (Simonović et al. 2011). A study by Kwon et al. is among the few preclinical studies investigating the consequences of combined exposure to mTBI and traumatic stress in animal models. Kwon et al. reported acute and transient anxiety in a group receiving unpredictable stress and a single blast injury relative to animals exposed to unpredictable stress alone (Kwon et al. 2011). In another study, Klemenhausen et al. found that repetitive concussive traumatic brain injury and post-injury foot shocks had synergistic effects in tests of social recognition and stress-coping behavior with no injury effects on recall of fear memories (Klemenhausen et al. 2013).

In the current study, cued fear memory recall was diminished in animals receiving repeated RUS and r-mTBI relative to RUS-only animals. Similar findings were observed at 3 and 6 months after a single exposure to RUS and/or r-mTBI (**Figure 3.4**). Genovese et al. reported similar findings showing an overall decrease in the expression of conditioned fear in rats exposed to inescapable electric shocks and blast overpressure injury relative to rats receiving shocks alone (Genovese et al. 2013). It is important to note that in our current study the injury was delivered 1 hour after RUS, whereas in the study by Genovese et al., the injury was delivered 22 hours after fear conditioning. One explanation for the mitigation of PTSD-related behavior when mTBI is applied post-exposure is that the memory of the foot-shock is erased due to the impact of TBI-dependent retrograde amnesia. Nevertheless, retrograde amnesia cannot completely explain our findings because stress+r-mTBI animals showed increased freezing behavior during the first minute of the

test at the 3-month timepoint (**Figure 3.9B-E**), suggesting that the memory of the cue is intact.

As demonstrated in **Figure 3.9**, stress+r-mTBI animals travelled longer distances in the OF test relative to animals in other study groups. This increase in their locomotor activity was negatively correlated with the immobility time in the cued fear memory test at the chronic timepoint (**Figure 3.10**). This correlation suggests that the increased locomotion in the comorbid group accounts for the decreased freezing in the cued fear memory test at the 3-month timepoint.

Increased locomotion in the open field has been attributed to behavioral disinhibition both in a mouse model of Alzheimer's disease (Tg2576 transgenic mice) (Gil-Bea et al. 2007) and in animals receiving 7 days of restraint stress (Ito et al. 2010). Interestingly, I have previously reported disinhibition or impulsive behavior in r-mTBI animals at 12 months after the last injury as demonstrated by increased time spent in the open arms and an increased number of entries into the open arms in the EPM (Mouzon et al. 2014).

I didn't observe a significant increase in open arm time or entries after r-mTBI in the current study possibly because the change in the lighting conditions. In our previous work by Mouzon et al., animals were tested in the EPM under bright light conditions, while in the current study animals were tested in the EPM in dark conditions (1 LuX). I choose to do these experiments in dark conditions, since these procedures facilitate observing the anxiety-like behavior. In bright conditions, control mice usually spend 5% - 10 % of the time in the open arms, which leaves very little margin to detect anxiety-like behavior.

The decreased freezing in the cued fear memory test in stress+rm-TBI animals compared to stress-only animals is most likely driven by r-mTBI-induced impulsive behavior or hyperactivity and caution should be used to avoid interpretation as strictly a deficit in cued fear memory recall. Likewise, the hyper-locomotion after r-TBI could also be responsible for the decreased immobility time in r-mTBI animals in the FST (**Figure 3.9I**) and (**Figure 3.7**) for the single exposure preliminary study. Similar observations were reported in Tg2576 transgenic mice showing a significant decrease in immobility time in the forced swim test that was inversely correlated with locomotor activity in the Open Field (Gil-Bea et al. 2007). There was no correlation between the distance travelled by stress+r-mTBI animals in the open field and their immobility time in the FST at the chronic timepoint (data not shown), suggesting that the observed FST behavior involves additional mechanisms. These results emphasize the importance of examining the effect of study treatments on locomotion before interpreting the findings of behavioral testing in rodents.

Peripheral inflammation has been implicated in the pathogenesis of TBI and PTSD with multiple reports of altered cytokine levels in the plasma (Acosta et al. 2013; Gill et al. 2009; Wang et al. 2017). I reported the following changes in baseline plasma cytokine levels at the chronic timepoint: IL-2 levels were reduced in all stress groups, IL-5 levels were reduced in all r-mTBI groups, and IL-10 levels were reduced in all stress and/or r-mTBI groups relative to the control group. IL-2 possesses a number of proinflammatory biological activities such as supporting the proliferation and survival of T cells, while IL-5 triggers the growth and differentiation of B cells and eosinophils (Kouro and Takatsu 2009). IL-10 is a potent anti-inflammatory cytokine that prevents the production of cell-mediated immunity or Th1 cytokines such as IL-2 and IFN- γ , and humoral immunity or

Th2 cytokines such as IL-4 and IL-5 (Mosser and Zhang 2008). Therefore, our findings may underlie an imbalance between Th1 and Th2 immune response after stress and r-mTBI. The impact of PTSD and mTBI on plasma cytokine levels in human patients is not clear. IL-2 levels were reduced in earthquake survivors and in middle-aged war veterans with PTSD (Jergović et al. 2014) relative to their age-matched healthy subjects. In contrast, veterans with PTSD had higher levels of salivary IL-2 relative to veterans without combat-related PTSD (Wang et al. 2016). Similarly, some reports showed decreased IL-10 levels in veterans with PTSD (Wang et al. 2016), while others showed increased IL-10 levels in PTSD patients relative to healthy controls (de Oliveira et al. 2018).

Some studies reported an increase in IL-6 and TNF α production in whole blood from PTSD patients after ex vivo stimulation with LPS (Gil 2010; Gola et al. 2013). I performed similar experiments on fresh blood collected from our study groups. The response to LPS stimulation appeared similar in all study treatments, although some interesting trends resembling the clinical findings were observed for TNF α and IL-6 in the stress-only group (**Figure 3.16**).

Spatial learning deficits after r-mTBI have been connected to changes in brain structures such as the hippocampus (Prasad and Bondy 2015). Hippocampal and PFC volume reduction and synaptic loss are also implicated in PTSD pathogenesis (Sherin and Nemeroff 2011). Multiple preclinical animal models reiterate the human findings and show the deleterious effects of stress and TBI on neuronal structures in the PFC and hippocampus (Ito et al. 2010; McEwen et al. 2016; Pitman et al. 2012; Wolf and Herringa 2016). In the current study, I focused on the hippocampus as a primary site for

pathobiology since both stress and r-mTBI seem to affect hippocampal-related functions such spatial and contextual memories.

Synapses are the junctions between neurons that facilitate communication and information processing in the brain. More than 90% of excitatory synapses are localized on the dendritic spines (Engert and Bonhoeffer 1999). Synaptic plasticity (long-term changes in the synapses in response to increased or decreased surrounding neuronal activity) is involved in the formation and retention of memories and is often accompanied by changes in the number and shape of dendritic spines (Frankfurt and Luine 2015; Li et al. 2004). Exposure to repeated RUS and r-mTBI alone or in combination decreased the number of dendritic spines in the CA1 of the hippocampus, but not in the PFC (**Figure 3.19**). Similar effects of stress and mTBI on hippocampal dendritic spines have been previously reported (Gao et al. 2011; Qiao et al. 2016; Shors, Falduto, and Leuner 2004; Winston et al. 2013). A study using 10 days of immobilization stress reported dendritic atrophy and debranching in the CA3 pyramidal neurons (Vyas et al. 2002), while another study using controlled cortical impact in mice showed a reduction in dendritic spines in the ipsilateral dentate gyrus at 24 (Winston et al. 2013) and 72 (Gao et al. 2011) hours after injury. Remodeling of hippocampal dendritic spines has implications for acquisition, consolidation and retrieval of spatial memories (Frankfurt and Luine 2015; Li et al. 2004; Luine, Wallace, and Frankfurt 2011; Mahmmoud et al. 2015). This was demonstrated in a study that found decreased spine density in the CA1 region of the hippocampus correlated with spatial memory deficits in an object placement task in aged rats (Luine et al. 2011).

Synaptic plasticity in the cortex and the hippocampus is primarily mediated by N-methyl-D-aspartate (NMDA) receptor signaling, which are a class of glutamatergic excitatory receptors (Engert and Bonhoeffer 1999; Ultanir et al. 2007). NMDA receptors

are tetrameric structures containing two essential GluN1 subunits and two GluN2 subunits (Sanz-Clemente, Nicoll, and Roche 2013). Age, brain region and memory consolidation are known to regulate the expression of different NMDA receptor subunits (Sanz-Clemente et al. 2013). At early developmental stages, GluN1 and GluN2B are the main components of NMDA receptors expressed at synapses. GluN2A replaces GluN2B as the primary GluN2 subunit with aging in what is known as developmental switch (Newcomer, Farber, and Olney 2000). Evidence suggests that the ratio of GluN2A to GluN2B increases with induced synaptic plasticity or memory consolidation in adult brains (Baez, Cercato, and Jerusalinsky 2018; Cui et al. 2013; Liu et al. 2007). At the 3-month timepoint, the ratio of hippocampal GluN2A/GluN2B was decreased in all study groups (**Figure 3.19H**), suggesting a chronic change in the maturity of synapses after stress and/or r-mTBI. In addition to the changes in GluN2, GluN1 levels were also reduced with stress and/or r-mTBI at the acute timepoint (**Figure 3.20**) and increased in stress+r-mTBI group at the chronic timepoint (**Figure 3.19**) relative to the control group. Behavioral and long-term memory deficits have been reported after knocking-down the GluN1 subunit in rat hippocampus by an amplicon vector expressing a specific GluN1-antisense RNA sequence (Adrover et al. 2003; Cercato et al. 2017; Cheli et al. 2006). Downregulation of hippocampal GluN1 levels at the acute timepoint could be attributed to an earlier plastic change in the hippocampus after RUS or r-mTBI, while the increased levels at the chronic timepoint may represent an adaptive homeostatic mechanism to restore GluN1 levels. Activation of GluN2A subunits has neuroprotective and cell survival effects, while activation of GluN2B appeared to have opposite effects (Song et al. 2007). In addition, treatment with NMDA receptor antagonist prevented stress-induced spine changes in the hippocampus of male and female rats (Shors et al. 2004). The chronic engagement of

NMDA receptors was also demonstrated at the level of downstream postsynaptic proteins CMKII α and PSD95 (**Figure 3.19**).

After a TBI, BDNF levels increase to lessen the impact of secondary injury, provide neuroprotection, and restore brain connectivity (Kaplan, Vasterling, and Vedak 2010). Increased BDNF levels has been reported in the cortex and the hippocampus of injured animals (Griesbach et al. 2002) and in the CSF of TBI patients (Failla, Conley, and Wagner 2016). In animal models of stress, prolonged exposure to glucocorticoids decrease BDNF levels in the hippocampus, causing dendritic retraction and remodeling (Kaplan et al. 2010). ELISA analysis of total BDNF levels in hippocampus homogenate did not show significant group differences. However, the BDNF precursor, ProBDNF, was upregulated in the hippocampus of r-mTBI-only animals at the 3-month timepoint. These findings support other reports showing increased BDNF signaling after mTBI. Interestingly, ProBDNF levels were not increased in the group receiving stress+r-mTBI, suggesting an opposite effect for stress and r-mTBI on BDNF signaling.

These results demonstrate that animals exposed to stressful trauma develop persisting traits that reflect critical aspects of PTSD symptomatology as defined by the DSM-5. Unique traits were also observed with the comorbid presentation of mTBI and trauma. The chronic timepoints examined here are particularly relevant to multiple persistent symptomology in the human condition. T anticipate that this model will be a useful platform to explore the neurobiology of PTSD and repetitive mTBI.

Chapter 4. Exploring the impact of RUS on HPA axis regulation and function

4. 1 Introduction

Physiological responses to stressful or life-threatening events involve the activation of the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. While the autonomic responses are immediate and transient, the HPA axis reaction starts several minutes after stress and is critical to restore homeostasis (Mifsud and Reul 2016) (MCEWEN 2004).

The paraventricular nucleus of the hypothalamus responds to stress by releasing corticotropin-releasing hormone (CRH) into the portal circulation. Binding of CRH to its receptor in the anterior pituitary results in the secretion of adrenocorticotrophic hormone (ACTH) which in turn stimulates the adrenal cortex to release cortisol (corticosterone in rodents) into the blood circulation. Circulating glucocorticoids exert several physiological functions to facilitate arousal during a stress response including, energy mobilization (e.g. gluconeogenesis) and maintenance of heart rate and blood pressure. In addition, glucocorticoids play a role in the acquisition of new memories and regulating homeostasis (Ulrich-Lai and Herman 2009). To avoid overactivation of the HPA axis, cortisol binds glucocorticoid receptors in the pituitary and the hypothalamus preventing further release of CRH and ACTH (Leistner and Menke 2018).

Mineralocorticoid receptor (MR) and Glucocorticoid receptor (GR) are the main binding sites for glucocorticoids in the brain. The affinity of glucocorticoids to MR is 10-fold higher than to GR in rodent brain tissue, and at baseline levels, glucocorticoids occupy

more than 80% of MR (Reul, van den Bosch, and de Kloet 1987). During exposure to stressful stimuli and HPA axis activation, whereby glucocorticoid levels become significantly upregulated, occupation of GR by glucocorticoids significantly increases (de Kloet, Oitzl, and Joëls 1993; De Kloet and Reul 1987; Reul et al. 1987). Thus, it is believed that MR mediates tonic actions (background activity) of glucocorticoids under physiological conditions, while GR mediates the negative feedback regulation mechanisms (De Kloet and Reul 1987).

Dysregulation of these feedback mechanisms is implicated in the pathogenesis of several psychiatric disorders such as depression and posttraumatic stress disorder (PTSD) (Leistner and Menke 2018). PTSD patients show increased CRH levels in cerebrospinal fluid (CSF) and decreased baseline plasma cortisol levels (Yehuda 2001). It is hypothesized that the often reported low baseline cortisol levels in PTSD patients are due to enhanced negative feedback sensitivity to glucocorticoids.(Yehuda et al. 1993, 1995). This hypothesis is supported by the increased sensitivity to dexamethasone (selective GR agonist) suppression reported in PTSD subjects (Yehuda et al. 1993). In addition, the GR co-chaperone FK506 binding protein 5 (FKBP5) was downregulated in the red blood cells and the brains of PTSD patients (Averill et al. 2017; Yehuda et al. 2009). As FKBP5 is known to play a significant role in regulating GR sensitivity, this finding supports the notion of impaired HPA axis negative feedback mechanisms in PTSD.

As detailed in Chapter 2, our newly developed PTSD mouse model demonstrates several chronic HPA axis alterations resembling human PTSD. These changes include low baseline plasma corticosterone and ACTH levels and reduced expression of FKBP5 in the hypothalamus at a chronic timepoint (Algamal et al. 2018). Our stress procedures relied on repeated unpredictable stress (RUS) that was followed by social isolation (SI), which is

a critical concurrent factor in patients known to increase vulnerability and risk for PTSD (Andrews, Brewin, and Rose 2003; Daskalakis, Yehuda, and Diamond 2013; Ullman and Filipas 2001; Zoladz et al. 2008).

Note: Our RUS paradigm, as described in Chapters 2 and 3 and Algamal et al. 2018, included SI as one of the stressors; however, as the work in this chapter delves into the separate effects of SI at this point I use RUS+SI when referring to the paradigm employed in Chapters 2 and 3, to distinguish from SI alone.

Here, I further explore the role of HPA axis regulation and function in our model at a sub-chronic timepoint, one month after RUS. The first goal of the study was to examine the function of two key components of the HPA axis, namely the role of the pituitary and adrenal glands using *in vivo* Dexamethasone/CRH and Dexamethasone/ACTH tests, respectively, in stressed mice (receiving RUS and SI) compared to controls (unstressed mice).

Secondly, I examine the functionality of central glucocorticoid receptor (GR) in RUS+SI mice under physiological and acute stress challenge conditions, with emphasis on negative feedback mechanisms involving FKBP5. In these experiments, as social isolation has been shown to be a major driver of HPA axis abnormalities in both clinical and preclinical studies (Hawkley et al. 2013), I included SI mice as an additional control group to address the contributory role of social isolation in our combined RUS+SI paradigm.

4. 2 Materials and Methods

Animals

C57BL/6 male mice (aged 9 weeks) were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed in standard cages (3 per cage) at ambient temperature. Mice were allowed to acclimate for three weeks before starting any experiments. All the procedures were carried out under a protocol approved by the Roskamp Institute IACUC (Protocol #91).

Experiment 1

This experiment involved two study groups; 1) Repeated unpredictable stress (RUS) in addition to social isolation group (RUS+SI group) and 2) group-housed controls (Control group).

Repeated unpredictable stress (RUS)

RUS procedures were implemented as described previously in Chapter 2 and in (Algamal et al. 2018). In brief, 12-week old mice in the RUS+SI group received daily unstable social housing with an unfamiliar mouse, 10 unpredictable exposures to predator odor (TMT) under restraint on 10 different days, and 5 inescapable foot-shocks in 5 on different days. RUS+SI animals were singly housed until the end of the study (Day 1-Day 56). Group-housed mice control mice (n=18) were conditioned to the trauma context and cues without receiving the foot-shocks.

Dexamethasone-CRH test

Baseline plasma corticosterone levels were measured 1 week before the actual test. On the test day (Day 56), control (n=18) or RUS+SI (n=22) mice were injected i.p. with 0.09 mg/kg, Dexamethasone (DEX (Sigma)) at 11:00 AM, followed by an i.p. injection of 0.2 mg/kg Corticotrophin Releasing Hormone (CRH (Sigma)) or Saline at 5:00 PM. A blood sample was collected from the tail vein (see below) immediately before CRH injection (i.e. 6 hours after injecting dexamethasone, (After DEX)). Another blood sample was collected 30 min after CRH injection for corticosterone and ACTH measurements (After CRH).

Dexamethasone-ACTH

The adrenal function was assessed in the same cohort of mice using the DEX-ACTH test. This test was performed 3 weeks after the DEX-CRH test and using similar procedures (Day 77). Animals were injected i.p. with 0.09 mg/kg DEX at 11:00 AM, followed by an i.p. injection of 0.1 mg/kg, ACTH (Sigma) or Saline at 5:00 PM. Blood samples were collected immediately before the injection of ACTH or saline (After DEX) and 30 min after ACTH injection (After ACTH) or Saline (After Saline).

Low dose Dexamethasone challenge

The same cohort of mice received a low dose dexamethasone suppression test 1 month after the DEX-ACTH challenge. Control and RUS+SI animals were injected i.p.

with 0.02 mg/kg DEX or saline at 11:00 AM, followed by a single blood collection at 5:00 PM.

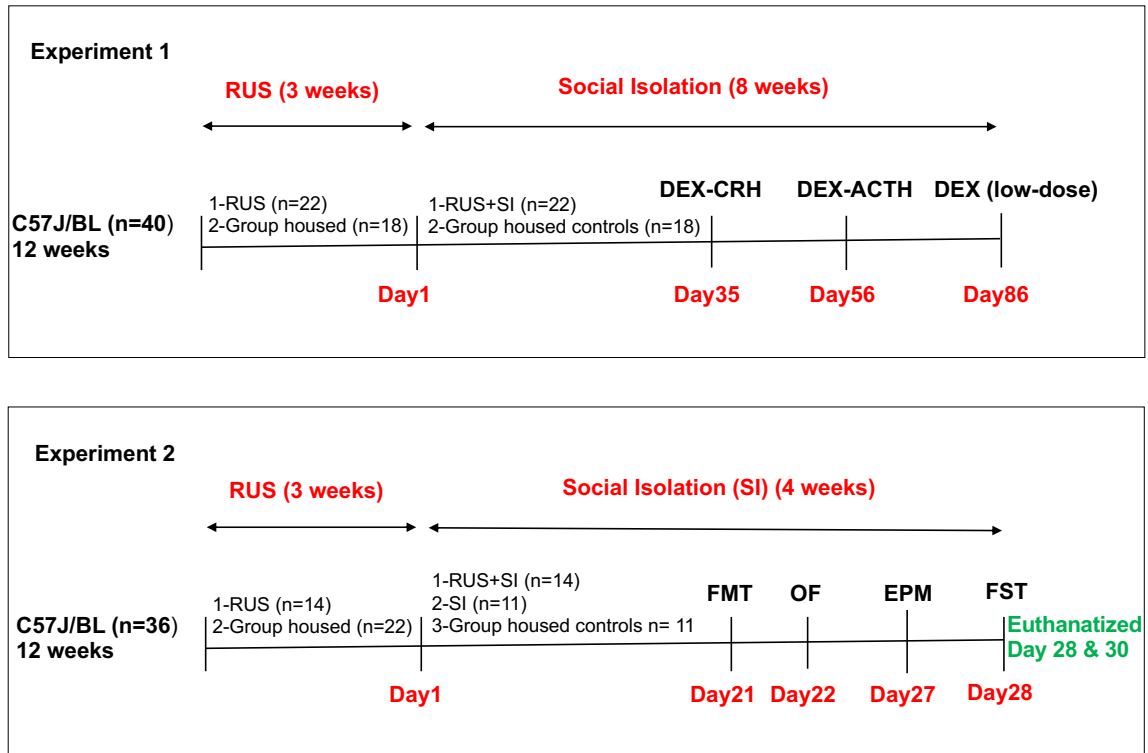


Figure 4.1. Timeline of the study procedures. In experiment 2, animals from different study groups were euthanized at 2 hours (Day 28) or 2 days (Day 30) after the FST. Abbreviations; RUS (Repeated unpredictable stress), Social Isolation (SI), DEX (Dexamethasone), Fear memory testing (FMT), Open Field (OF), Elevated Plus Maze (EPM), and the forced swim test (FST).

Experiment 2

Experiment 2 was performed on a new independent cohort of mice as depicted in **Figure 4.1**. The experiment involved three study groups; 1) RUS in addition to social isolation group (RUS+SI group), 2) social isolation-only group (SI group) and 3) group-

housed controls (Control group). RUS procedures were implemented as described in Experiment 1 and Chapter 2. After exposure to RUS, animals were singly housed until the end of the study (Day 1-Day 30). Group-housed mice (n=22) were conditioned to the trauma context and cues without receiving the foot-shocks, as described in experiment 1. On day 1 of the study timeline (i.e., at the end of the RUS procedures), 11 mice out of the 22 previously group housed Control mice were singly housed until the end of the study (SI group), while the other 11 remained undisturbed (Control group).

Behavioral testing

Behavioral testing started at day 21 after the stress/control paradigm as depicted in **Figure 4.1**. Mice received contextual and cued fear memory testing (FMT), Open Field test (OF), Elevated Plus Maze (EPM) and the Forced Swim Test (FST) followed by euthanasia at either 2 hours (n=5-6) or 2 days after the FST (n=5-6). All the behavioral procedures were conducted as described previously (Algamil et al. 2018).

Acute stress reactivity in the periphery

The FST was the last behavioral test in the study timeline and was additionally used to examine the effect of acute stress on HPA axis regulation. Blood samples were collected 13 days before (Baseline) and 15 min after the FST (Response) by tail bleed. Corticosterone recovery after stress was measured in blood samples from animals euthanatized at 2 hours after the start of the FST (Recovery).

Blood collection

The tail bleed procedures involved collecting a few blood drops (10-20 μ L) from a small incision in the lateral tail vein at days 14 (Baseline) and 28 (Response). All tail bleed procedures were performed in a room next to the housing room and completed in less than 5 minutes. Blood samples from euthanized animals were collected by cardiac puncture after 3 min of isoflurane anesthesia. All blood samples were centrifuged at 3,000g for 5 min, and plasma samples (clear supernatant fraction) were flash frozen in liquid nitrogen and stored at -80°C .

ELISA

All ELISA kits were used as per manufacturers' instructions. The Adrenocorticotrophic Hormone (ACTH) ELISA was purchased from Cloud-Clone Corp (Katy, Texas), while the Corticosterone ELISA was purchased from Arbor Assays (Ann Arbor, Michigan).

Brain tissue preparation and isolation of nuclear and cytoplasmic fractions

To examine the regulation of glucocorticoid receptor after an acute stressor, animals were euthanized and brain and plasma were collected at either 2 hours or 2 days after the FST. Brain tissue collected 2 hours after the FST was used to examine the acute stress-related changes in protein expression levels (n=5-6 per group), while tissue collected 2 days after the FST was used to examine the recovered-baseline expression levels of relevant markers (n=5-6 per group,).

Animals were transferred to the euthanasia room at least 90 min before sacrifice to allow for HPA axis recovery after transfer. After anesthesia and blood collection by cardiac perfusion, brains were extracted and dissected into pituitary, hypothalamus, and hippocampus and immediately flash frozen in liquid nitrogen and kept at -80°C .

To isolate the nuclear and cytoplasmic enriched fractions from the hypothalamus, tissue was weighed and homogenized in Phosphate Buffered Saline (PBS) containing proteinase and phosphatase inhibitors (PPI) (Thermofisher) using a probe sonication. 6 μL of PBS+PPI were added to each 1 mg of tissue. 50 μL of homogenized samples were mixed with equal volumes of PBS+PI containing 1.5M NaCl and spun at 21,000 g for 10 minutes (min) followed by the collection of the supernatants containing the cytosolic and nuclear fractions. To separate the enriched nuclear fraction, 25 μL methanol was added to the supernatant solution under gentle vortexing followed by vigorous vortexing and 15 min incubation on ice. Samples were then spun at 21,000 g for 15 min followed by collection of the supernatants (containing the cytosolic fraction) and the pellet (nuclear fraction). The nuclear fractions were obtained by resuspending the pellets in 100 μL (25 mM Triethylammonium bicarbonate (TEAB) containing 0.05% sodium Deoxycholate (SDC)). The cytosolic enriched fractions were obtained by mixing the remaining supernatant solution with 325 μL methanol under gentle vortexing followed by vigorous vortexing and 15 min incubation on ice. Samples were then spun at 21,000 g for 15 min followed by discarding the supernatant and collecting the pellet (cytosolic fraction) which was then dissolved in 100 μL 25 mM TEAB with 0.05% Deoxycholat (SDC). The pituitary was homogenized directly in 100 μL of M-PER protein extraction reagent without fractionation, due to the small amount of starting material.

Western blotting

Western blot experiments were conducted as described previously (Ojo et al. 2016). In brief, homogenized or fractionated samples were centrifuged at 15,000g for 10 minutes (min) and tissue supernatants were collected. Supernatant fractions were denatured at 99 °C by boiling in Laemmli buffer (Bio-Rad) containing dithiothreitol (DDT). Samples were subsequently resolved on 4-15% gradient polyacrylamide criterion gels (Bio-Rad). After electro-transferring, polyvinylidene difluoride membranes, the membranes were blocked in 5% milk made in Tris-buffered saline and subsequently immunoprobed for different brain-specific primary antibodies overnight (Supplementary Table 1). After three washing steps, membranes were probed with horseradish peroxidase-linked secondary antibodies (Supplementary Table 1). Bio-Rad stain-free technology was used to quantify total protein for the hypothalamic fractions, while Anti-GAPDH antibody was used as a housekeeping protein and loading control for the pituitary lysates. Signal intensities were quantified by chemiluminescence imaging with the ChemiDoc™ XRS (Bio-Rad).

Statistical Analysis

The choice of the appropriate statistical test was made after assessment of normal distribution using the Shapiro-Wilk normality test. RM-Two-way ANOVA was used to examine statistical significance in body weight measurements, DEX-CRH and DEX-ACTH tests. All western blot analysis and baseline corticosterone measurements in Experiment 1 were analyzed using One-Way ANOVA. All ANOVA were followed by pairwise comparisons with correction for multiple comparisons using the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (BKY) (Benjamini, Krieger, and

Yekutieli 2006) to control the false discovery rate. All analyses were performed with Graph Pad prism version 7.0 statistical software (La Jolla, California). Grubbs' test was applied to identify statistical outliers.

4. 3 Results

Experiment 1. RUS+SI mice show attenuated response to CRH stimulation in the DEX-CRH test.

Dexamethasone (DEX) is a synthetic glucocorticoid acting mainly on GR located in the anterior pituitary, with limited availability in other brain regions due to rapid clearance by multidrug resistant transporters (Meijer et al. 1998). At a sufficient dose, DEX will bind GR in the pituitary, thereby suppressing the release of ACTH which in turn will result in a decrease in circulating plasma corticosterone levels. To examine the HPA axis negative feedback regulation at the level of the pituitary, I performed the dexamethasone-CRH test. At one month after RUS, baseline plasma corticosterone levels were lower in RUS+SI mice relative to the group housed controls (t-test: $t_{(39)} = 2.9$, $p = 0.006$, **Figure 4.2A**). The DEX-CRH test was administered one week after the collection of baseline values. Mice were given a high dose of dexamethasone (0.09 mg/kg) at 11:00 am in the morning. Six hours after DEX injections, plasma corticosterone levels were equally suppressed in control and RUS+SI animals as compared to their baseline levels ($p > 0.05$, **Figure 4.2B**). Immediately after this blood collection (6 hours after DEX), CRH was injected intraperitoneally followed by another blood collection 30 min later to determine the response to this DEX-CRH test. RUS+SI animals showed significantly lower plasma corticosterone levels compared to controls (RM-Two-Way ANOVA: interaction $F_{(1, 38)} = 4.195$, $p = 0.047$, main CRH effect $F_{(1, 38)} = 452.4$, $p < 0.0001$ main RUS+SI effect $F_{(1, 38)} = 3.95$, $p = 0.05$ with correction for multiple comparisons, **Figure 4.2B**), and no significant change in plasma ACTH levels (t-test, $p > 0.05$, **Figure 4.2C**). Because ACTH levels were normal after the challenge, this suggests that the pituitary response to CRH stimulation is similar in control and RUS+SI animals.

Experiment 1. RUS+SI mice show attenuated response to ACTH stimulation in the DEX-ACTH test.

Mice were left undisturbed for three weeks after receiving the DEX-CRH test and then received the DEX-ACTH test (Eiler and Oliver 1980). The purpose of the test is to assess the response of the adrenal glands to exogenous ACTH after the suppression of endogenous ACTH and corticosterone with DEX. The same dexamethasone dose used in the DEX-CRH test was applied to suppress endogenous ACTH and corticosterone levels followed by injection of 0.1mg/kg ACTH or Saline after 6 hours. Plasma corticosterone levels were equally suppressed in control and RUS+SI animals 6 hours after DEX injection ($p>0.05$, **Figure 4.6D**). 30 min after ACTH injection, plasma corticosterone levels were significantly elevated in both groups in response to ACTH stimulation. However, the adrenal response in RUS+SI animals was significantly attenuated as demonstrated by lower plasma corticosterone levels in the RUS+SI group relative to control at 30 min after ACTH injection (RM-Two-Way ANOVA: interaction $F_{(1, 17)} = 11.94$, $p=0.003$, main ACTH effect $F_{(1, 17)} = 413.4$, $p<0.0001$, main RUS+SI effect $F_{(1, 17)} = 6.35$, $p=0.022$ with correction for multiple comparisons, **Figure 4.2D**). Saline injection also caused a pronounced increase in corticosterone levels after dexamethasone suppression (RM-Two-Way ANOVA: interaction $F_{(1, 17)} = 2.19$, $p=0.164$, main Saline effect $F_{(1, 17)} = 78.77$, $p<0.0001$, main RUS+SI effect $F_{(1, 17)} = 1.9$, $p=0.18$ with correction for multiple comparisons, **Figure 4.2E**). However, the overall increase in corticosterone levels after saline injection was two-fold lower than the ACTH-induced increase, thus suggesting that the administered ACTH dose was primarily responsible for the stimulation of the adrenal glands.

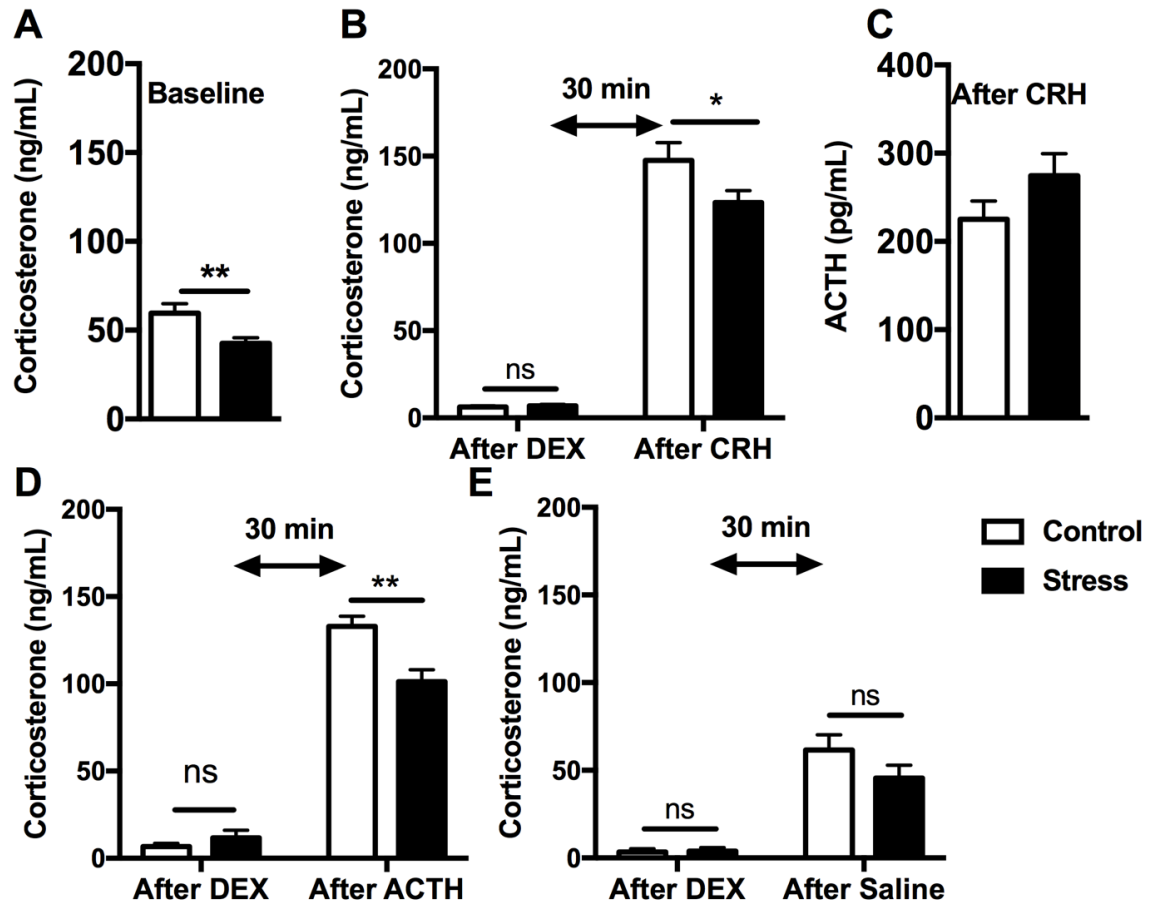


Figure 4.2. Decreased HPA axis responsiveness after CRH and ACTH stimulation in stressed animals. The DEX-CRH stimulation test was administered 5 weeks after RUS. (A) Baseline plasma corticosterone levels measured 1 week before the DEX-CRH stimulation test. (B) Plasma corticosterone levels 6 hours after suppression with DEX (After DEX) and 30 min after a subsequent stimulation with CRH (After CRH). (C) Plasma ACTH levels 30 min after CRH stimulation in the DEX-CRH stimulation test. The same group of mice were used in the DEX-ACTH stimulation test at 9 weeks after RUS. (D) Plasma corticosterone levels 6 hours after suppression with DEX (After DEX) and 30 min after a subsequent stimulation with ACTH (After ACTH). (E) Same as (D) except for the use of saline injections after DEX suppression instead of ACTH. In A-C, $n=18-22$, while in D-E, $n=6-11$. All Data were analyzed using a student t-test. Asterisks denote statistical significance as follows * $p<0.05$, ** $p<0.01$.

Experiment 1. Low dose DEX suppresses plasma corticosterone levels in RUS+SI mice and control mice.

One month after administering the DEX-ACTH challenge, control and RUS+SI received the low-dose dexamethasone suppression test (0.02mg/kg as compared to the 0.09mg/kg of the previous tests). Control and RUS+SI mice showed comparable corticosterone suppression levels at 6 hours after i.p. injection of 0.02 mg/kg DEX (Two-Way ANOVA: interaction $F_{(1, 32)} = 0.05$, $p=0.8214$, main DEX effect $F_{(1, 32)} = 8.41$, $p=0.0067$, main RUS+SI effect $F_{(1, 32)} = 2.773$, $p=0.11$ with correction for multiple comparisons, **Figure 4.3**.

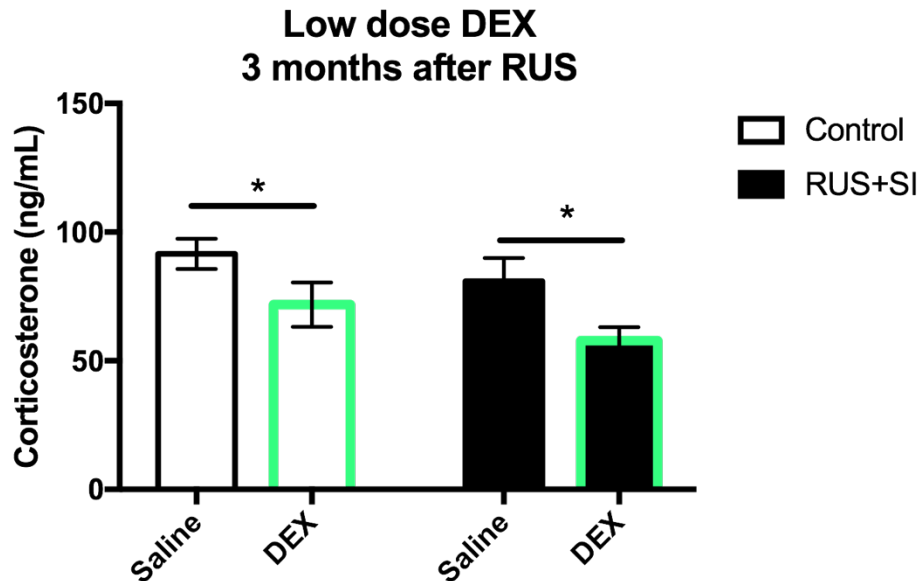


Figure 4.3. Performance of each study group in the low-dose dexamethasone suppression test. Plasma corticosterone levels in control and RUS+SI mice 6 hours after an injection of saline or 0.02 mg/kg DEX. Bar graphs depicting control and RUS+SI mice receiving saline are outlined with black lines, while bar graphs depicting control and RUS+SI mice receiving DEX have green border lines. Asterisks denote statistical significance as follows * $p<0.05$.

Experiment 2. Decreased basal and stress-induced plasma corticosterone levels in socially isolated and RUS mice.

PTSD patients show low plasma cortisol levels in response to the Trier Social Stress Test (TSST), which examines the reactivity to social and psychological stress under laboratory settings in humans (Pierce and Pritchard 2016; Wichmann et al. 2017). To examine the stress-reactivity in RUS+SI mice, I measured plasma corticosterone levels in response to an acute heterotypic stressor (forced swim). I also assessed stress reactivity in mice receiving only SI to examine if our findings can be reproduced by SI alone, as the role of SI in our paradigm remains undetermined. All groups showed a stress response as demonstrated by increased corticosterone levels 20 min after initiating the FST relative to their respective baseline values (Two-Way ANOVA: interaction $F_{(4, 74)} = 1.25$, $p > 0.05$, main acute stress effect $F_{(2, 74)} = 45.43$ with correction for multiple comparisons, **Figure 4.4A**). Plasma corticosterone levels were lower in SI and RUS+SI mice relative to control mice at baseline and 20 minutes (Response) after the forced swim test (Two-Way ANOVA: interaction $F_{(4, 74)} = 1.25$, $p > 0.05$, main treatment effect $F_{(2, 74)} = 5.01$, $p = 0.009$ with correction for multiple comparisons, **Figure 4.4A**). All groups showed similar corticosterone levels at 2 hours after the FST (Recovery) (Two-Way ANOVA, $p > 0.05$). Recovery ACTH levels showed a trend towards increase in the RUS+SI mice relative to control and SI groups (One-way ANOVA, $F_{(2, 11)} = 3.53$, $p = 0.065$, with correction for multiple comparisons, **Figure 4.4B**).

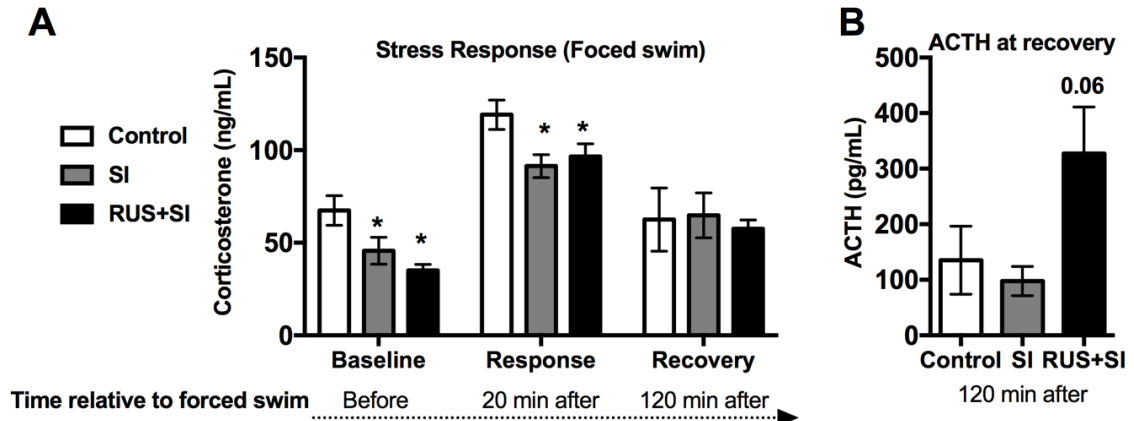


Figure 4.4. Effect of RUS and social isolation on reactivity to acute stress. (A) Baseline plasma corticosterone levels were measured 10 days before the forced swim test (Baseline). Plasma corticosterone levels were measured 20 min (Response) and 120 min (Recovery) after initiating the forced swim. (B) Plasma ACTH levels 120 min after the cessation of forced swim (Recovery). Data in A was analyzed using Two-Way ANOVA followed by the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli (BKY) to control the false discovery rate, while data in B was analyzed using One-Way ANOVA. $n=11-14$ in baseline and stress response measurement while $n=5-6$ in all Recovery measurements. Asterisks denote statistical significance as follows $*p<0.05$.

Experiment 2. Social isolation and RUS differentially regulate stress-induced expression of HPA axis markers in the hypothalamus

I have previously shown the RUS+SI mice show a downregulation in the GR regulatory protein FKBP5 at 6 months after RUS (Algamil et al. 2018). To better understand GR functionality in RUS+SI and SI mice under physiological condition and following HPA axis activation, I measured the protein expression levels of the GR and its associated proteins in the hypothalamus and the pituitary in response to acute stress challenge. Animals euthanized at 2 hours and 2 days after the FST were used to examine the stress-induced and baseline expression levels of relevant HPA axis markers, respectively. Activation of GR involves the translocation of the receptors from the cytoplasm to the nucleus followed by binding to glucocorticoid responsive elements (GRE)

to activate gene expression (Fries, Gassen, and Rein 2017). Hence, I obtained the nuclear and cytoplasmic fractions from the hypothalamus in order to examine the effect of acute stress on GR translocation in RUS+SI mice. In each fraction, the expression levels of GR, FKBP and CRH were measured at baseline, and after acute stress, using western blotting. No changes were observed in the cytoplasmic GR levels at baseline or in response to acute stress or treatment ($p>0.06$, **Figure 4.5A**), although there was a trend towards increased cytoplasmic GR levels in RUS+SI animals relative to control and SI animals. In the hypothalamic nuclear fraction, baseline levels of GR were lower in RUS+SI relative to controls. In addition, nuclear GR levels were higher in all groups in response to acute stress with the relative levels after stress being lower in SI and RUS+SI animals compared to controls (Two-Way ANOVA: interaction $F_{(2, 27)} = 1.863$ $p=0.18$, main acute stress effect $F_{(1, 27)} = 52.6$, $p<0.001$ main treatment effect $F_{(2, 26)} = 5.14$, $p=0.012$ with correction for multiple comparisons, **Figure 4.5D**).

FKBP5 levels were reduced in the cytoplasmic fraction of SI mice relative to controls and RUS+SI mice after acute stress (Two-Way ANOVA: interaction $F_{(2, 26)} = 2.78$, $p=0.984$, main acute stress effect $F_{(1, 26)} = 0.67$, $p=0.41$ main treatment effect $F_{(2, 26)} = 4.3$, $p=0.023$ with correction for multiple comparisons, **Figure 4.5B**). Additionally, nuclear FKBP5 levels were down regulated in control and RUS+SI mice after acute stress, while it remained elevated in SI mice after acute stress (Two-Way ANOVA: interaction $F_{(2, 26)} = 0.87$, $p=0.42$, main acute stress effect $F_{(1, 26)} = 9.237$, $p=0.0052$ main treatment effect $F_{(2, 26)} = 0.1$, $p=0.9$ with correction for multiple comparisons, **Figure 4.5E**).

Two-way ANOVA showed no changes in the hypothalamic cytoplasmic and nuclear levels of CRH at baseline and in response to acute stress at the examined timepoint ($p>0.05$, **Figure 4.5C** and **4.5F**). However, examining the group differences after acute

stress using One-way ANOVA showed that nuclear CRH levels were reduced in the RUS+SI group relative to SI after acute stress (One-way ANOVA, $F_{(2, 13)} = 6.31$, $p = 0.012$, with correction for multiple comparisons, **Figure 4.5F**)

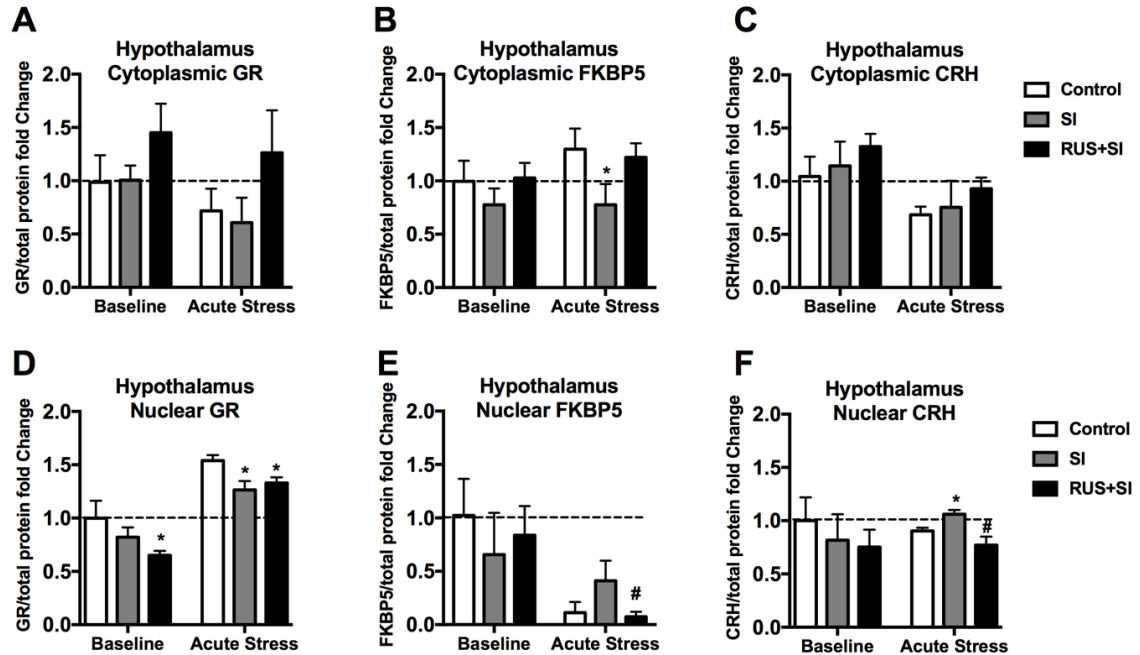


Figure 4.5. Effect of acute stress on HPA axis regulation in the hypothalamus at 1 month after RUS. Quantification of western blot images in the cytoplasmic and nuclear enriched fractions in response to acute stress in the hypothalamus. A, B, and C depict the effect of acute stress on cytoplasmic levels of GR, FKBP5, and CRH, respectively, while D, E, and F depict the effect of acute stress on nuclear levels of GR, FKBP5, and CRH, respectively. Data were analyzed using One-Way ANOVA followed by two-stage linear step-up procedure of BKY to control the false discovery rate ($n = 5-6$). Statistically significant findings versus the control group are denoted by *, while statistically significant findings versus the SI group are denoted by #.

Experiment 2. Social isolation and RUS differentially regulate stress-induced expression of HPA axis markers in the pituitary

I then examined the expression levels of MR, GR, FKBP5, CRH, and its type1 receptor CRH-R1 in whole cell lysates from the pituitary. Cellular fractionation of pituitary could not be achieved due to the small tissue size. No changes were observed in pituitary GR levels at baseline or in response to acute stress ($p>0.05$, **Figure 4.6A**). FKBP5 levels were downregulated in the RUS+SI group relative to the SI group at baseline and relative to the control group after acute stress (Two-Way ANOVA: interaction $F_{(2, 24)} = 2.78$, $p=0.08$, main acute stress effect $F_{(1, 24)} = 5.4$, $p=0.0289$ main treatment effect $F_{(2, 24)} = 4.19$, $p=0.027$ with correction for multiple comparisons, **Figure 4.6B**). No significant change was observed in Two-way ANOVA of pituitary CRH and or its receptor CRH-R1 levels. However, One-Way ANOVA showed increased CRH-R1 levels in the RUS+SI group relative to control and SI groups after acute stress (One-way ANOVA, $F_{(2, 11)} = 9.162$, $p=0.0045$, with correction for multiple comparisons, **Figure 4.6D**). No differences between study groups were observed in the pituitary levels of GR, MR or CRH at one month after RUS (Two-Way ANOVA, $p>0.05$, **Figure 4.6**).

I also examined the effect of acute stress on high affinity glucocorticoid receptors (MR) in the pituitary. After acute stress, the levels of pituitary MR were downregulated to the same levels, across all groups relative to their baseline values, with no difference between the groups at either baseline or in response to acute stress ($p>0.05$, **Figure 4.6E**).

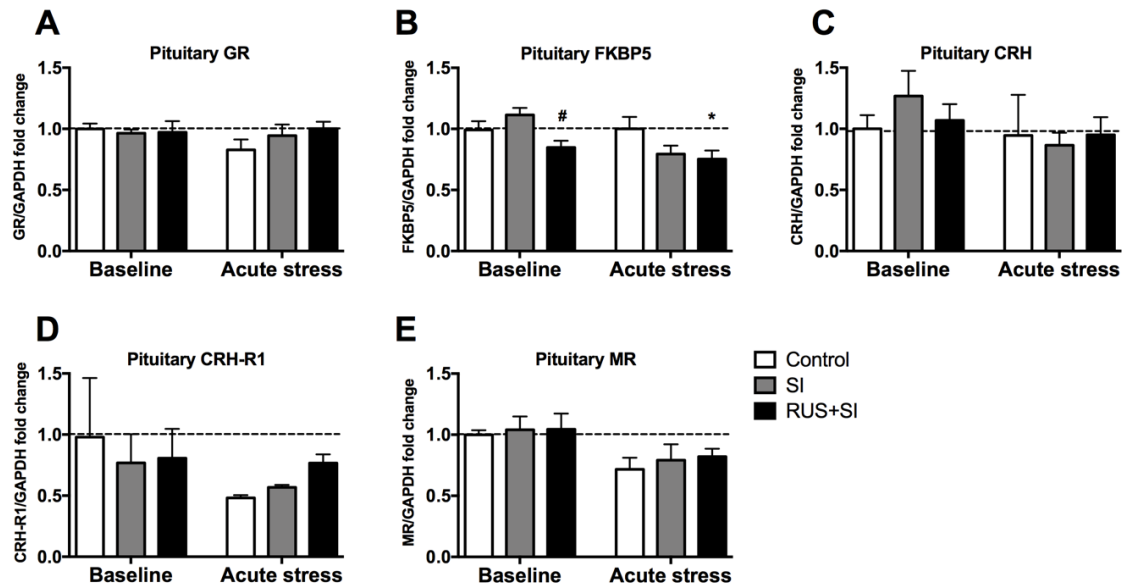


Figure 4.6. Effect of acute stress on HPA axis regulation in the pituitary of RUS and SI mice. Quantification of pituitary GR (A), FKBP5 (B), CRH (C) (CRH-R1) (D) and MR (E) levels at baseline and after acute stress using western blotting. Data were analyzed using One-Way ANOVA followed by two-stage linear step-up procedure of BKY to control the false discovery rate ($n=4-6$). Statistically significant results versus the control group are denoted by *, while statistically significant results versus the SI group are denoted by #.

Experiment 2. Body weight deficits after exposure to RUS or chronic social isolation.

Socially isolated mice (SI) showed decreased body weight relative to the control group after 27 days of social isolation, while animals exposed to RUS in addition to social isolation (RUS+SI) showed decreased body weight at the end of the RUS paradigm (Day 1), which persisted after 27 days of social isolation) (RM-ANOVA: interaction $F_{(8, 128)} = 5.14$, $p < 0.0001$, with correction for multiple comparisons, **Figure 4.7A**). RUS+SI and SI mice showed similar bodyweights at Day 27 ($p > 0.05$, **Figure 4.7A**).

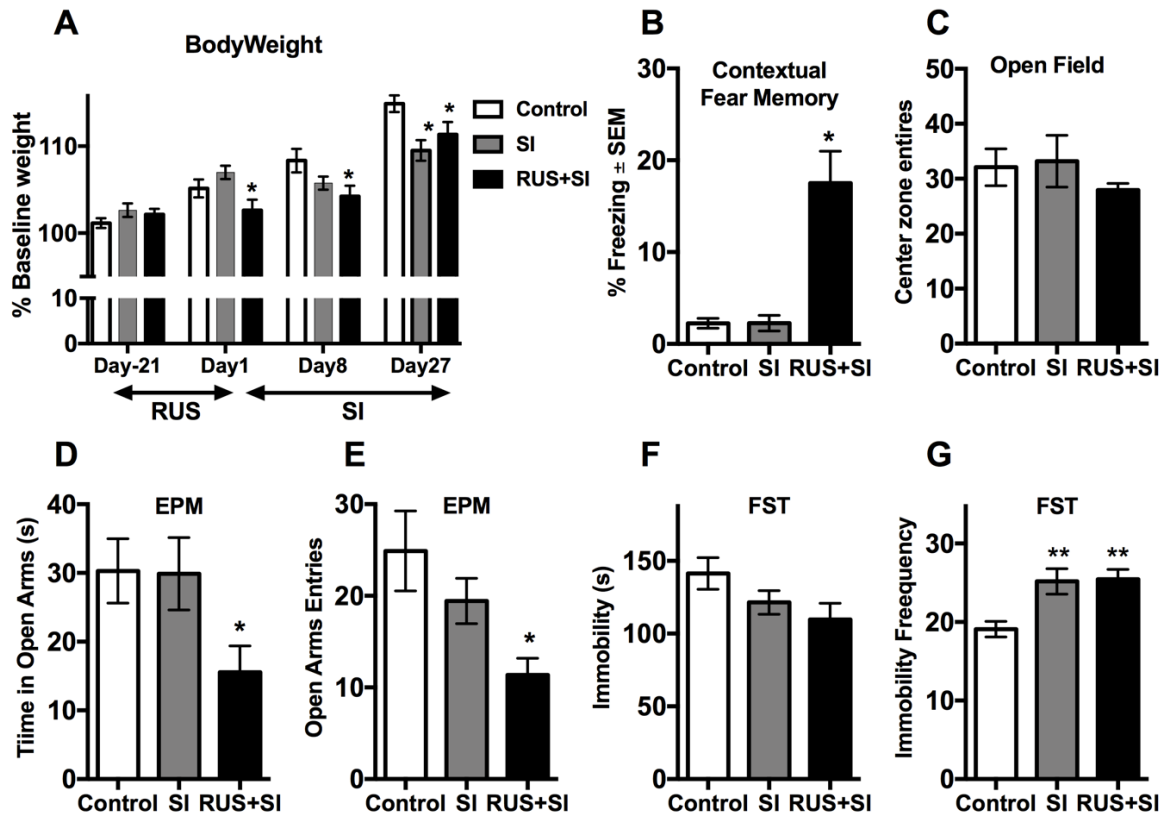


Figure 4.7. Effect of RUS and social isolation on body weight, anxiety and stress-coping behavior. Changes in mice bodyweight relative to baseline after RUS and SI (A). Performance of each group in the contextual fear memory test (B). Anxiety-like behavior was assessed by examining the number of entries to the OF center zone (C), total time spent in open arms of the EPM (D), and the number of entries to the open arms of the EPM (E). Immobility time and immobility frequency in the FST are depicted in panels (F) and (G), respectively. Data in A was analyzed using RM-Two-way ANOVA, while data in (B-G) were analyzed using One-Way ANOVA followed by two-stage linear step-up procedure of BKY to control the false discovery rate. (n=11-14). Statistically significant findings versus the control group are denoted by *.

Experiment 2. Combined exposure to RUS and social isolation is associated with anxiety-like behavior one month after RUS.

In our previous characterization of the RUS paradigm, recall of contextual fear memories and anxiety-like behavior was demonstrated at an acute timepoint, but not at the 3-months or the 6-months timepoints (Chapter 2 and (Algamil et al. 2018)). Our findings

at the chronic timepoints were not conclusive, because the same cohort of mice was subjected to retesting at all timepoints. In the current study, all behavioral testing was performed once at approximately one month after RUS (Day 21 to Day 28 of the study timeline).

RUS +SI mice showed increased % freezing time in the trauma context relative to control and SI mice (One-Way ANOVA, $F_{(2, 33)} = 14.29$, $p < 0.0001$, with correction for multiple comparisons, **Figure 4.7B**). To examine the chronic effects of RUS on anxiety, I performed the Open Field (OF) and Elevated Plus Maze (EPM) tests at one month after RUS without any prior testing at Day 42. As depicted in Figure 4.7, avoidance behavior was observed only in the stress group (RUS+SI) as evident by decreased open arm time (One-way ANOVA, $F_{(2, 27)} = 3.58$, $p = 0.041$, with correction for multiple comparisons, **Figure 4.7D**), and open arm entries (One-way ANOVA, $F_{(2, 27)} = 5.28$, $p = 0.012$, with correction for multiple comparisons, **Figure 4.7E**). No changes were observed in the total distance travelled (F) or not-moving time (H) in stressed animals relative to controls (One-way ANOVA, $p > 0.05$, Figure S1C and S1D). No change in the FST immobility time was observed between study groups (One-way ANOVA, $p > 0.05$, **Figure 4.7F**). However, the immobility frequency was increased in stress and SI animals relative to the control group suggesting that they share similar stress coping strategies (One-way ANOVA, $F_{(2, 30)} = 7.5$, $p = 0.002$, with correction for multiple comparisons, **Figure 4.7G**).

4. 4 Discussion

Imbalance in HPA axis regulation is implicated in a number of affective disorders including PTSD in which it often presents as low baseline plasma cortisol levels (Deslauriers et al. 2018; Pitman et al. 2012; Yehuda 2001). In this Chapter, I explored the mechanisms of HPA axis regulation in our PTSD mouse model. As previously described in Chapter 2, this model has been extensively characterized at 6 months after exposure to repeated unpredictable stress (RUS). Here, I performed studies at the earlier timepoint of one month after RUS, where I anticipated the demonstration of persistent and prominent subchronic behavioral abnormalities in our model and a suitable time-window for assessing theragnostic effects in our model in anticipation of future studies exploring novel therapeutic approaches in drug discovery.

A number of explanations for the blunted HPA axis after PTSD have been proposed including enhanced negative feedback sensitivity at the level of the pituitary (Yehuda et al. 1993). I examined the pituitary negative feedback regulation mechanisms in RUS+SI mice in vivo using the DEX-CRH test. DEX was first given to suppress endogenous release of ACTH since DEX acts mainly on the pituitary. As a result of ACTH suppression, circulating glucocorticoids levels decreased accordingly. CRH was then injected to stimulate the pituitary to release ACTH which in turn increased the synthesis and release of glucocorticoids. Decreased plasma glucocorticoid levels in response to CRH stimulation may indicate a hypofunctional pituitary or adrenal gland and can also arise due to increased sensitivity of GR to dexamethasone. Control and RUS+SI animals showed similarly suppressed corticosterone levels after a high dose dexamethasone, and lower corticosterone levels in response to CRH stimulation in RUS+SI animals (**Figure**

4.2B). Low corticosterone levels after CRH stimulation can be attributed to impaired pituitary or adrenal functions. However, the normal or slightly elevated ACTH levels in RUS+SI animals after CRH stimulation suggested that the low plasma corticosterone concentration cannot be explained by pituitary hypofunction (**Figure 4.2C**). If the pituitary function were impaired due to enhanced sensitivity to dexamethasone suppression or decreased overall ability to release ACTH, plasma ACTH levels should have been lower in RUS+SI mice after CRH stimulation. Therefore, these findings suggested that the adrenals are unresponsive to ACTH stimulation. To further check this hypothesis, I retested the same cohort of mice in the combined DEX-ACTH stimulation test. Similarly, a high dose DEX was given to prevent the release of any endogenous ACTH from the pituitary followed by ACTH or saline injection. Consistent with our hypothesis, RUS+SI animals showed decreased plasma corticosterone levels after ACTH stimulation relative to the group-housed controls (**Figure 4.2D**). Similar findings have previously been observed in a study examining the effects of chronic subordinate colony (CSC) housing- a form of psychosocial stress- on adrenal function (Reber et al. 2007). In this study, adrenal glands isolated from CSC mice failed to respond to multiple doses of ACTH stimulation in vitro (Reber et al. 2007).

Alternatively, these findings can also be explained by increased sensitivity to dexamethasone suppression at the level of the adrenal glands. Recent evidence has shown that GR are expressed in the adrenal cortex in rodents and humans (Walker et al. 2014). This finding suggests that glucocorticoids may be able to autoregulate their own synthesis and release via intra-adrenal mechanisms (Walker et al. 2014). Therefore, in our study, increased sensitivity of adrenal GR to dexamethasone may also account for the decreased corticosterone release in RUS+SI animals in the combined DEX_ACTH test.

High or low doses of DEX alone (**Figure 4.2 and 4.3**) were not associated with a significant change in HPA axis negative feedback sensitivity in RUS+SI animals relative to control animals. Retesting the same cohort of mice in the HPA challenges is one of the limitations of this study, due to the possibility of inducing long-term plastic changes in the HPA axis after DEX and CRH administration (Touma et al. 2011). The DEX dose examined here before prior testing is high and so may have been efficient in completely suppressing corticosterone levels in all groups. Additional studies using lower DEX doses in an independent cohort of mice are needed to clearly elucidate the sensitivity of GR to negative feedback regulation.

Group-housing of laboratory mice is recommended and widely used to accommodate the social nature of rodents (Kappel, Hawkins, and Mendl 2017). Single-housing or social isolation is considered a form of psychosocial stress in mice because of its impact on behavior, and the physiological and neuroendocrine systems (Arndt et al. 2009). Early-life, juvenile or adult social isolation for a period of 2 to 8 weeks is associated with changes in HPA axis activity and stress-coping strategies (Boero et al. 2018; Gądek-Michalska et al. 2017; Pisu et al. 2016). Specifically, adult socially isolated mice show lower plasma corticosterone levels at baseline and higher levels in response to acute stress when compared to group housed control mice (Berry et al. 2012).

In this study, socially isolated (SI) mice and RUS+SI mice showed similar changes in bodyweight, stress coping strategies and corticosterone stress response to acute stress. However, the abundance of GR and FKBP5 in the brain in response to acute stress was markedly different between RUS+SI and SI mice, indicating that the mechanisms regulating GR activity are different in both groups. FKBP5 is a part of an ultra-short negative feedback loop that regulates the GR response to acute stress (Hubler and

Scammell 2004; Paakinaho et al. 2010). Under baseline conditions (normal corticosterone levels), GR is held in the cytoplasm as a multiprotein complex containing the co-chaperones FKBP5 and heat shock protein 90 (HSP90). After acute stress, corticosterone levels increase and bind to cytoplasmic GR resulting in its transport to the nucleus after dissociation from chaperone proteins. Activation of GR transcriptional activity by its binding to the glucocorticoid response element (GRE) in the nucleus increases the expression of FKBP5 and other regulatory proteins to block additional GR translocation (Merkulov, Merkulova, and Bondar 2017). Although the involvement of cytoplasmic FKBP5 in translocation of GR is well established, little is known about the function of nuclear FKBP5 (Brkic et al. 2017). Some reports have suggested a role for nuclear FKBP5 in increasing GR stability in the nucleus by protecting non-DNA-bound-GR from proteolysis (Tatro et al. 2009).

Baseline GR levels were lower in RUS+SI animals relative to control mice in the nuclear fraction of the hypothalamus, suggesting a difference in subcellular localization of GR in our model (**Figure 4.5D**). This is also supported by the trend towards higher baseline levels of cytoplasmic GR in RUS+SI mice relative to control and RUS mice (**Figure 4.5A**). No change was observed in the subcellular baseline levels of FKBP5 in the hypothalamus suggesting that other mechanisms are involved. These mechanisms may involve changes in other chaperone proteins such as FKBP4 and HSP9 and may also underlie deficiencies in GR export from the nucleus. Interestingly, previous reports have shown that elevated FKBP4/FKBP5 ratio in the hippocampal nuclear fraction was associated with increased GR nuclear localization(Tatro et al. 2009).

In response to acute stress in the Forced Swim Test, levels of GR were lower in both RUS+SI and SI mice relative to controls, while FKBP5 levels were higher in SI mice

relative to group housed controls in the hypothalamic nuclear fraction. I am unable to determine whether the decrease in nuclear GR was mainly due to alteration in nuclear translocation, as FKBP5 levels would have appeared higher in the cytoplasmic fraction. Given that cytoplasmic FKBP5 levels were lower in SI animals and unchanged in RUS+SI animals, this implicates interplay of other unknown regulatory mechanisms.

A limitation of the current study is the analysis of only a single timepoint after acute stress (2 hours after forced swim stress). Additional experiments using multiple earlier timepoints are needed to clearly understand the exact mechanisms involved here. However, these results clearly suggest that that stress adaptation mechanisms are different in RUS+SI and SI animals. Interestingly, anxiety-like behavior was only observed in animals exposed to RUS in addition to SI at one month after RUS (**Figure 4.7**). This finding also strengthens the validity of our chronic PTSD model, since the persistence of anxiety-like behavior could not be established in our previous characterization of the RUS model in Chapter 2.

Here, I have shown that exposure to the RUS stress paradigm resulted in a number of HPA axis alterations including decreased plasma corticosterone concentrations in response to acute stress, DEX-CRH stimulation, and DEX-ACTH stimulation. In addition, the model also shows changes in GR subcellular localization in the hypothalamus of stressed mice implicating that GR sensitivity and HPA axis feedback are altered. The HPA axis alterations in this model, although not perfectly resembling human PTSD, are unique to an animal model of stress and will be valuable in understanding the complexity and intricate details of the HPA axis response to stress. Because of the heterogenous nature of PTSD, our findings may be relevant to the fraction of PTSD patients showing low baseline glucocorticoids levels accompanied by a slight alteration in pituitary function. In

agreement with previous reports (Leistner and Menke, 2018), my findings emphasise the importance of examining HPA axis function in response to acute stress to better engage GR. Finally, I show that while social stress is a key factor for the persistence of symptoms in this model, the findings cannot be solely reproduced by social isolation stress.

Chapter 5. Effect of dexamethasone and/or 7,8 dihydroxyflavone treatment on stress-related behavior in the RUS model

5. 1 Introduction

The lack of progress in the psychopharmacologic treatment of post-traumatic stress disorder (PTSD) is an ongoing crisis due to its negative clinical, social and economic implications (Krystal et al. 2017). Current PTSD pharmacotherapy relies on a few FDA approved depression medications which offer only symptomatic relief and show limited efficacy (Krystal et al. 2017; McAllister 2009). As both military and civilian populations are increasingly exposed to traumatic stress, the identification of effective etiology-based treatments for the condition is a high priority. As presented in Chapter 1, valid preclinical PTSD animal models, showing long-lasting PTSD-like traits, are invaluable in that respect because they enable fast and efficient testing of potential drugs.

In Chapter 2 and Chapter 4, I showed that exposure to repeated unpredictable stress (RUS) and subsequent social isolation were effective in demonstrating several chronic PTSD-related phenotypes in a mouse model. These chronic traits were observed in multiple systems, most noticeably in the HPA axis and BDNF signaling. The HPA axis changes included decreased plasma corticosterone levels at baseline and after acute stress (**Figure 2.8 and 4.4**), altered GR subcellular localization in the hypothalamus (**Figure 4.5**), and increased pituitary CRH-R1 levels in response to acute stress (**Figure 4.6**). In addition, hippocampal BDNF levels were significantly reduced at 6 months after RUS (**Figure 2.10**) and possibly at 10 days after RUS (A trend towards reduction, **Figure 2.14**).

Here I examine if pharmacological targeting of these two systems individually or in combination would be effective in restoring homeostasis in stressed animals. Because hippocampal BDNF levels were downregulated 6 months after RUS, I used the orally available BDNF receptor (TrkB) agonist 7, 8-dihydroxyflavone (DHF) to restore BDNF signaling. To target the HPA axis, I used a low dose dexamethasone (DEX) for a month after RUS. DEX is a corticosterone analogue with higher receptor affinity and limited central availability, therefore treatment with a low DEX dose should be mainly supportive for the peripheral glucocorticoid function. Interestingly, a single low dose of DEX was effective in accelerating the extinction of fear memories in humans and rodents with mechanism involving altered GR sensitivity in the amygdala during fear extinction (Michopoulos et al. 2017; Sawamura et al. 2016). Therefore, this study will also examine the consequences of chronic administration of low-dose DEX on fear memory recall and other on stress-related behavior to further validate our model. DEX and DHF were administered alone or in combination in drinking water for a month followed by behavioral testing and target engagement studies.

5. 2 Materials and Methods

Animals

I used C57BL/6 male mice aged 12 weeks old at the time of exposure to RUS to investigate the impact of our therapeutic interventions on stress-related behaviors. The RUS procedures were implemented as described in Chapter 2 followed by a month of drug administration in drinking water starting at day 3 after RUS. As depicted in **Figure 5.1**, the experiment involved one vehicle treated control group (C-VEH, n=10) and 4 stress groups (n=9-10 per group) receiving either vehicle (S-VEH), Dexamethasone-only (S-DEX), DHF-only (S-DHF), or a combination of both (S-DEX+DHF).

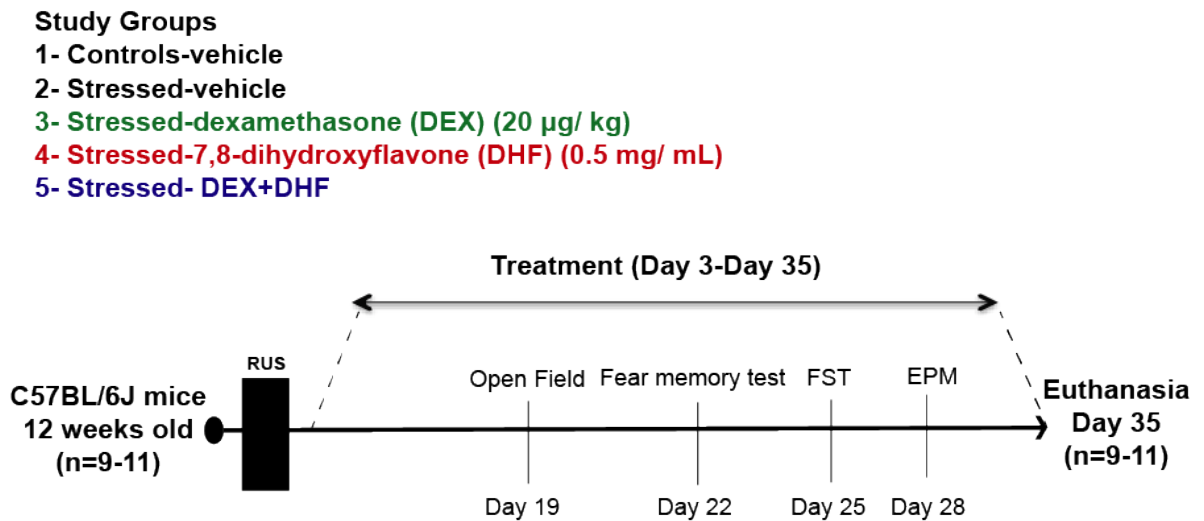


Figure 5.1. Study timeline. Treatment started 3 days after the end of the RUS procedures. All animals were euthanized after \cong one month of treatment.

Drugs

7, 8-dihydroxyflavone (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in 100% dimethyl sulfoxide DMSO (Sigma) to generate a stock solution at 500

mg/mL. The stock solution (1 ml) was gently added to 1000 ml of water that contained 1% sucrose and sonicated for 30 min. The final concentration of DHF given to mice in drinking water was 0.5 mg/mL. Water soluble dexamethasone was purchased from Sigma and dissolved directly into the vehicle solution (1% sucrose, 0.1% DMSO) to a final concentration of (0.12 µg/mL) in drinking water. Estimating that each animal drinks 5-7 mL per day, this concentration approximates 20-40 µg/kg for 15-18-week-old mice. The drinking water containing either DHF, DEX, DEX+DHF, or vehicle was replaced every 2 days. Water bottles were weighed in each replacement cycle to estimate the average water consumption by each animal. The dose of DHF examined here was adapted from previous studies showing improved cognitive functions using a dose of 1 mg/mL in mouse models of psychosis and fragile X syndrome (Han et al. 2016; Tian et al. 2015). However, due to the immediate precipitation of DHF when dissolved at a concentration of 1mg/mL, the dose was reduced to 0.5 mg/mL followed by the sonication procedures described earlier to improve DHF vehicle solubility. These procedures resulted in a clear to slightly turbid DHF solution that was subsequently given to mice as drinking water. Several studies reported a much lower dose (0.08 mg/ mL) to be also effective in rodent models of stress, TBI and Rett Syndrome (He et al. 2016; R. A. Johnson et al. 2012; Sanz-García et al. 2016). To ensure that DHF treatment was associated with maximum TrkB receptor activation, I used the maximum reported, yet soluble, dose of DHF I can prepare.

Behavioral testing.

Behavioral assessment started 19 days after RUS, while mice were still receiving treatments, and included the Open Field test, contextual and cued fear memory testing, the forced swim test (FST) and the Elevated Plus Maze (EPM) (**Figure 5.1**). Animals were

tested in the FST twice with a 48 hours interval. All behavioral procedures were implemented as described previously in Chapter 2.

Euthanasia

Animals were euthanized 7 days after the last behavioral test (Day 35). The collection of plasma and brain tissue was followed as described previously in Chapter 2 and as previously published (Algamal et al. 2018).

Statistics

Freezing scores in the cued fear memory tests and the immobility time in the forced swim test were analyzed using Repeated measure Two-Way ANOVA. All other experiments were analyzed using One-Way ANOVA. All ANOVA experiments were followed by Two stage linear set up procedure of Benjamini, Krieger and Yekutieli (BKY) (Benjamini et al. 2006) to correct for multiple comparisons by controlling the false discovery rate ($q=0.05$). Grubb's test was used to identify outliers.

5.3 Results

DHF supplementation reverses anxiety-like behavior in stressed animals.

The average weekly intake of drinking water was monitored to ensure that all animals were receiving similar drug dosages. The weekly water consumption for C-VEH mice was lower than all animals in the stress groups, with no significant changes across stressed groups regardless of treatment (**Figure 5.2**) (RM-Two-Way ANOVA: main treatment effect $F(4, 42) = 6.88$, $p=0.0002$, with correction for multiple comparisons, **Figure 5.2A**). Treatment had no effect on body weight, since all stressed animals had lower bodyweights at euthanasia relative to the C-VEH group (One-Way ANOVA, $F(4, 40) = 4.22$, $p=0.006$, with correction for multiple comparisons, **Figure 5.2B**).

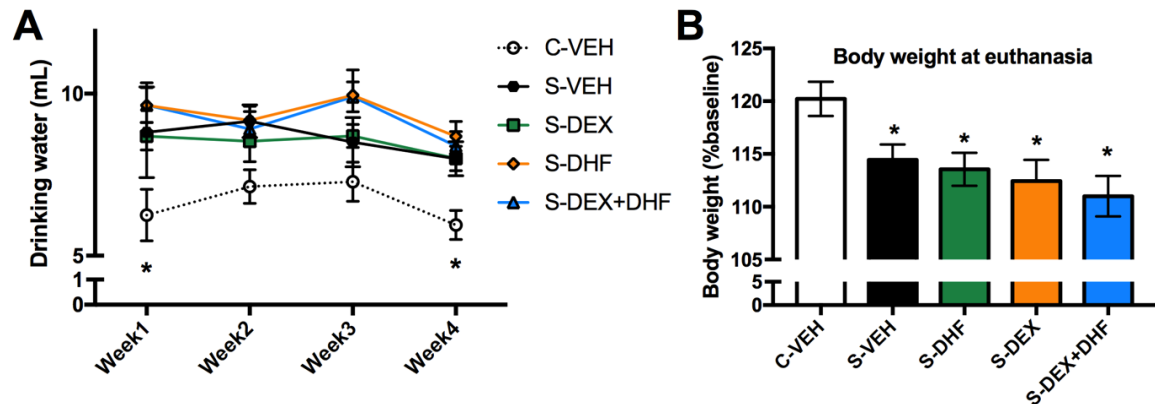


Figure 5.2. The average weekly intake of drinking water by each treatment group. (A) Average weekly drinking water consumption in each treatment group. (B) Body weights (% baseline) after one month of treatment (i.e. at euthanasia). Data in A was analyzed using RM-Two-Way ANOVA, while data in B was analyzed using One Way ANOVA.

S-VEH animals showed anxiety-like behavior as demonstrated by decreased center zone entries relative to C-VEH animals in the Open Field test (One-Way ANOVA, $F(4, 41) = 4.96$, $p=0.0024$, with correction for multiple comparisons, **Figure 5.3B**). DHF treatment reversed the anxiety-like behavior observed in S-VEH group, since entries to the Open Field center zone were not statistically significant between S-DHF or S-DHF+DEX animals and C-VEH animals. In addition, S-DHF+DEX group showed increased center zone entries relative to S-VEH animals suggesting that combined DEX and DHF are possibly more effective. DEX treatment did not reverse the anxiety-like behavior in stressed animals, because both S-DEX and S-VEH animals showed a decrease in Open Field center zone entries relative to C-VEH. Immobility time in the open field test was increased only in S-VEH animals relative to C-VEH (One-Way ANOVA, $F(4, 40) = 4.62$, $p=0.0037$, with correction for multiple comparisons, **Figure 5.3C**). Therefore, all treatment groups seem to reduce Open Field immobility time. All stressed animals showed decreased locomotor activity in the open field relative to C-VEH animals with no drug effects observed (One-Way ANOVA, $F(4, 40) = 8.335$, $p<0.0001$, with correction for multiple comparisons, **Figure 5.3D**). No change was observed in the time spent in the open field center zone between different study groups (One-Way ANOVA, $p=0.45$, **Figure 5.3A**).

I also assessed anxiety behavior in the EPM test. No significant treatment effects were observed in time spent in the open arms (One-Way ANOVA, $p > 0.05$, **Figure 5.3E**), entries to the open arms (One-Way ANOVA, $p > 0.05$, **Figure 5.3F**), number of nose dips (One-Way ANOVA, $p > 0.05$, **Figure 5.3G**), or the total distance travelled in the EPM (One-Way ANOVA, $p > 0.05$, **Figure 5.3H**). Although there was a trend towards reduced number of nose dips in the open space in S-VEH animals relative to C-VEH, no significant

differences were found in One-Way ANOVA analysis. This was probably attributed to the high variability in animals' performance in the EPM.

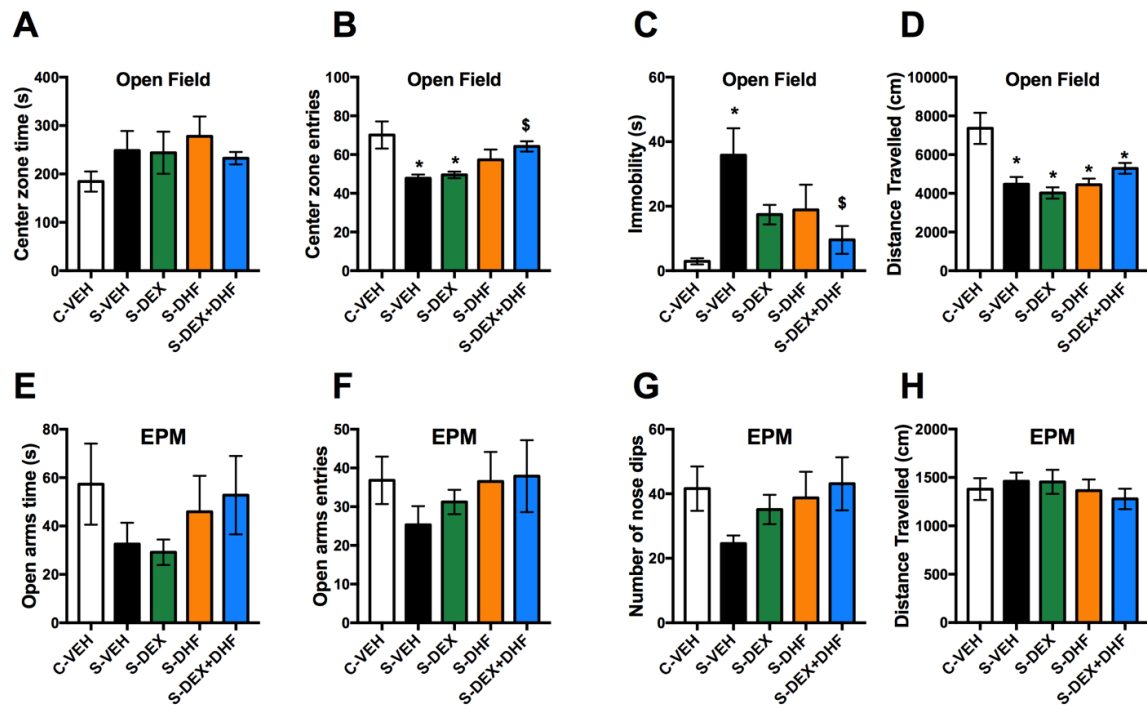


Figure 5.3. Effect of DEX and/or DHF intake on anxiety-like behavior after one month of treatment. Anxiety-like behavior was assessed in the Open field test at Day 19 after RUS and in the Elevated Plus Maze at Day 28 after RUS. Average time spent in the Open Field center zone is depicted in (A), while the number entries to center zone area is depicted in (B). The total immobility time and distance travelled in the Open Field are depicted in C and D, respectively. The time spent the EPM open arms, number of entries to the EPM, number of nose dips in the EPM the open area and total distance travelled in the EPM are depicted in E, F, G, and H, respectively. Data were analyzed using One-Way ANOVA followed by BKY method to correct for multiple comparisons. Asterisks (*) denote statistically significant results relative to the C-VEH group, while (\$) denote statistically significant results relative to the S-VEH group.

DEX intake decreases freezing response to the fear conditioning context.

The effect of DEX and/or DHF treatment on fear memory recall was examined at one month after RUS as depicted in **Figure 5.1**. DEX treatment attenuated contextual fear

memory recall as evident by increased freezing times in S-VEH and S-DHF animals relative to C-VEH, S-DEX, and S-DEX+DHF animals in the contextual fear memory test (One-Way ANOVA, $F(4, 40) = 5.77$, $p=0.0009$, with correction for multiple comparisons, **Figure 5.4A**). All treatment groups showed minimal freezing when placed in a novel context (No cue) 1 hour after the contextual fear memory test ($p>0.05$, **Figure 5.4B**). A tone (Cue) was turned on after 3 min of habituation in the new context to examine the cued fear memory response. All stress groups showed increased freezing time relative to the control group after tone introduction (RM-Two-Way ANOVA: interaction, $F(4, 40) = 13.62$, $p<0.0001$ main treatment effect $F(4, 40) = 10.99$, $p<0.0001$, with correction for multiple comparisons, **Figure 5.4B**). Interestingly, S-DEX animals showed reduced freezing time relative to S-VEH animals after tone introduction (**Figure 5.4B**).

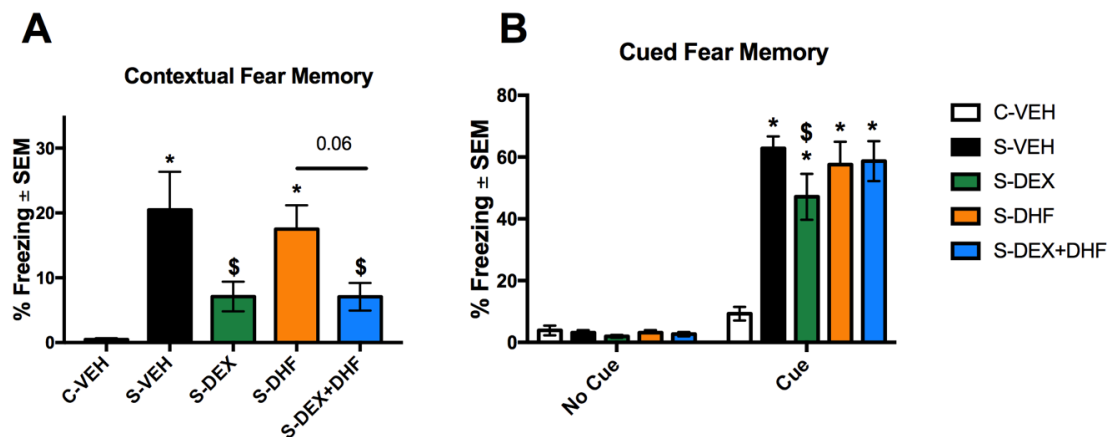


Figure 5.4. Effect of DEX and/or DHF intake on recall of fear memories after one month of treatment. The performance of each treatment group on contextual (A) and cued (B) fear memory tests. Data in A was analyzed with One-Way ANOVA, while data in B was analyzed with RM-Two Way ANOVA. Asterisks (*) denote statistically significant results relative to the C-VEH group, while (\$) denote statistically significant results relative to the S-VEH group.

All treatment groups showed similar stress coping behavior when examined in the FST (RM-Two-Way ANOVA, $p>0.05$, **Figure 5.5**).

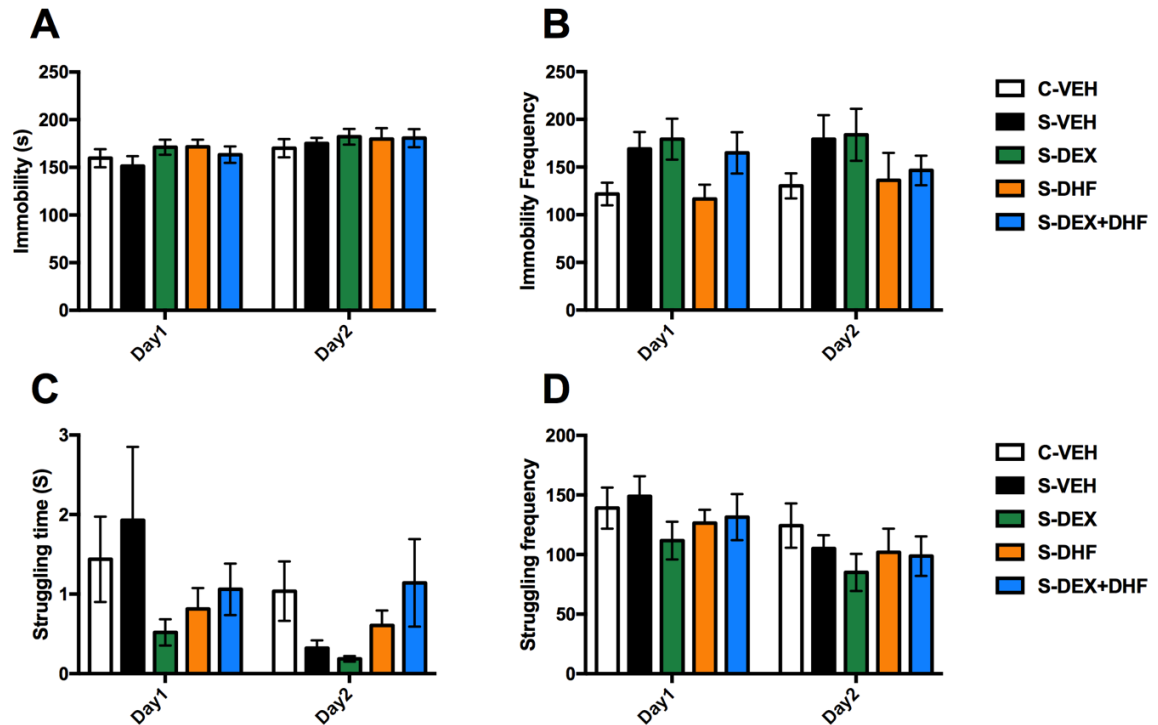


Figure 5.5. Effect of DEX and DHF intake on stress coping strategies. The performance of each treatment group in the forced swim test is depicted in **Figure 5.5** as follows; (A) immobility time, (B) immobility frequency, (C) struggling time, and (D) struggling frequency. RM-Two Way ANOVA was used to analyze the data.

Effect of DEX and/or DHF on baseline plasma corticosterone levels and TrkB phosphorylation in the hippocampus

To examine the effects of chronic DEX administration (20 $\mu\text{g}/\text{kg}$) on HPA axis suppression, I measured baseline plasma corticosterone levels at euthanasia. Baseline plasma corticosterone levels were lower in S-DEX and S-DEX+DHF animals relative to C-VEH and S-VEH animals (One-Way ANOVA, $F(4, 41) = 7.76$, $p<0.0001$, with

correction for multiple comparisons, **Figure 5.6.6B**). Surprisingly, S-VEH animals showed higher baseline corticosterone levels relative to C-VEH animals ($p=0.0156$, **Figure 5.6B**) in contrast to what I have reported previously. Plasma corticosterone levels in control animals at euthanasia appeared to be lower than to their levels prior to vehicle administration (**Figure 5.6A**). No change in the levels of P-TRKB or BDNF were observed in the hippocampus of all treatment groups ($p>0.05$, **Figure 5.7**).

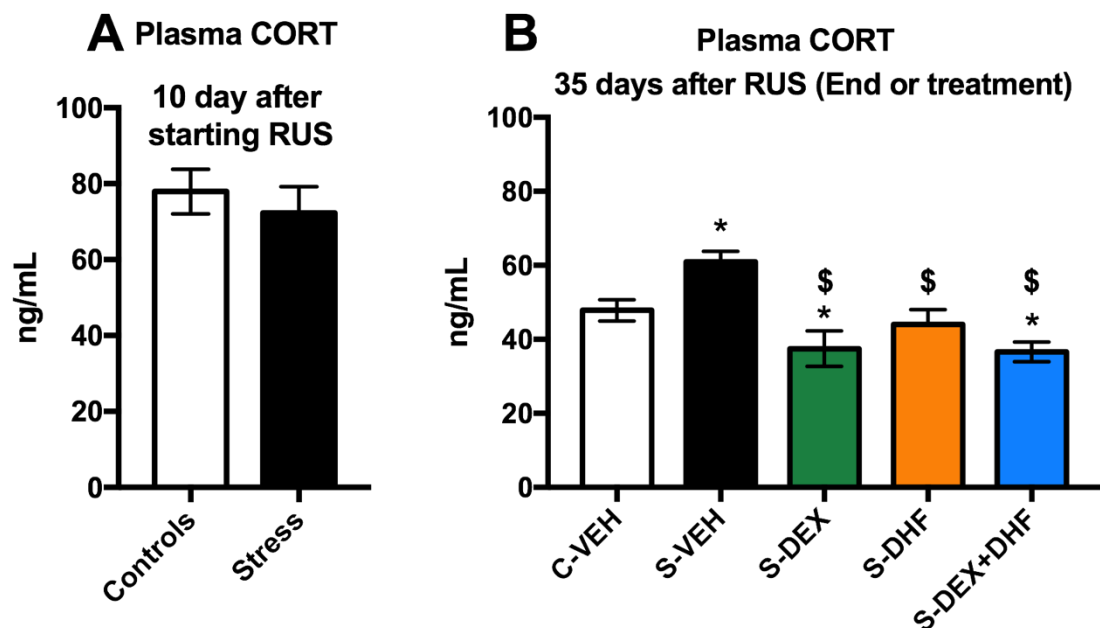


Figure 5.6. Effect of DEX and DHF intake on baseline corticosterone levels. A blood sample was drawn from control and stressed animals by tail bleed at day 10 of the ongoing RUS procedures and before treatment. (A) Pre-treatment baseline corticosterone levels 10 days after the start of the RUS paradigm (B) Baseline plasma corticosterone levels at euthanasia (i.e. after one month of treatment). Data in A was analyzed using a student test, while data in B was analyzed using One-Way ANOVA followed by BKY procedures to correct for multiple comparisons. Asterisks (*) denote statistically significant results relative to the C-VEH group, while (\$) denote statistically significant results relative to the S-VEH group.

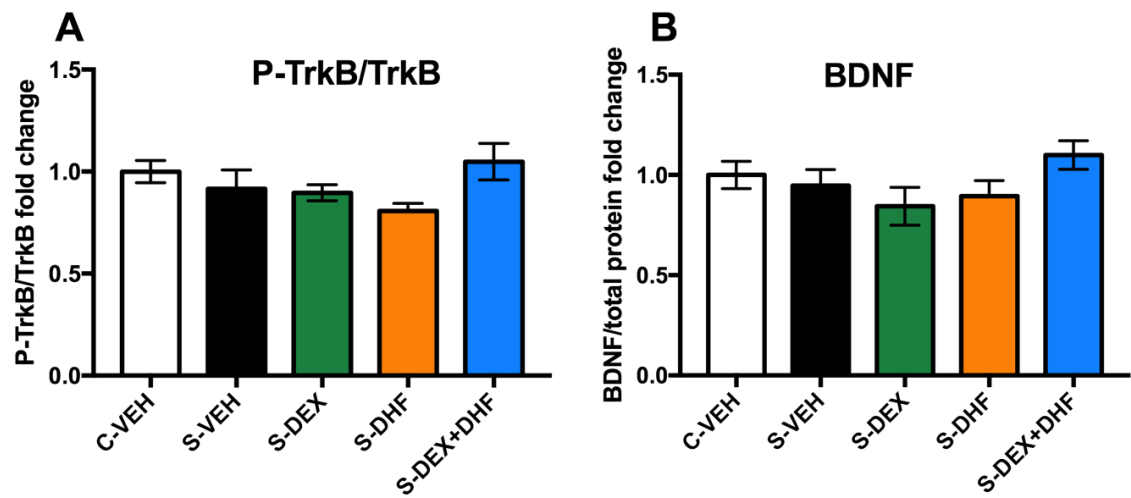


Figure 5.7. Effect of treatment on P-TrkB and BDNF levels in the hippocampus. Quantification of western blot images for P-TrkB (A), and BDNF from the hippocampus. Data was analyzed using One-Way ANOVA.

5. 4 Discussion

Herein, I examined the effects of combined treatment with an HPA axis regulator (DEX) and a TrkB receptor agonist (DHF) on neurobehavioral outcomes in mice at one month after RUS. Chronic DHF intake was associated with improvements in anxiety-like behavior and no effect on body weight or recall of fear memories. On the other hand, chronic DEX intake was associated with reduced freezing response to the trauma context and no effect on body weight, or anxiety-like behavior. These findings show beneficial outcomes for a combination therapy regulating the HPA axis and BDNF signalling.

A single dose of DEX has been previously demonstrated in humans to facilitate fear extinction and discrimination in PTSD patients when administered the night before a fear extinction session (Michopoulos et al. 2017). In mice, injection of a low DEX dose 4 hours before cued fear memory recall trials was associated with enhanced fear extinction (Sawamura et al. 2016). In addition, the DEX-induced changes in fear extinction was dose-dependent and may have involved epigenetic regulation of FKBP5 expression in the amygdala (Sawamura et al. 2016)

In the current experiment, chronic administration of DEX attenuated freezing response in the contextual and cued fear memory test relative to the vehicle treated stressed mice. These findings suggest that DEX effects on fear extinction are maintained after chronic low dose administration and therefore provide further validation to our model. The low DEX dose used here was effective in suppressing the HPA axis (**Figure 5.6**) relative to vehicle treated stressed mice. Interestingly, DHF alone was also effective in reducing corticosterone levels relative to stressed animals receiving vehicle suggesting that DHF effects may be partially mediated by HPA axis adjustments. The interaction between

the BDNF/TrkB system and HPA axis is well-established (Chen et al. 2012; Daskalakis et al. 2015; Jeanneteau et al. 2012). Intracerebroventricular BDNF injections upregulates CRH expression in the hypothalamus and increases plasma corticosterone concentrations (Givalois et al. 2004; Naert et al. 2006). In addition, increased glucocorticoids levels due to acute or chronic stress is known to downregulate BDNF levels and decrease TrkB phosphorylation in the hippocampus (Kunugi et al. 2010). In Chapter 2, I showed that BDNF and corticosterone levels were decreased in the hippocampus at 6 months after RUS. It is important to note that corticosterone levels were most likely elevated during the RUS procedures and possibly during the early days of social isolation. Therefore, the negative alterations in the hippocampus in our model, including reduced BDNF levels, can be possibly be attributed to initial elevation in corticosterone levels.

DHF has shown therapeutic potential in several animal studies of BDNF-related disorders such as Alzheimer's disease (Liu, Chan, and Ye 2016), Parkinson's disease (Jang et al. 2010), psychosis (Han et al. 2016), Rett syndrome (R. A. Johnson et al. 2012), and depression (Chang et al. 2016). In the context of PTSD, administration of DHF 8 hours prior to a spatial memory task prevented the immobilization-induced memory deficits in rats (Sanz-García et al. 2016). The reversal of the stress-associated behavior in the open field test by DHF (supported also by similar trends in the EPM) indicates that DHF administration partially recovers the unstressed phenotypes. Interestingly, polymorphism in BDNF gene (Val66Met) resulting in deficits in its secretion from neurons have been previously reported to increase anxiety-like behavior in mice (Chen et al. 2007). No change was observed in p-TrkB or BDNF levels in whole hippocampal homogenates, so activation of TrkB receptor under the current experimental procedures could not be confirmed. Additional screening for different TrkB phosphorylation sites in the hippocampus and

other brain regions, particularly in isolated fractions such as synaptosomes, and activation of downstream signaling targets, is required to thoroughly explore target engagement of DHF in the brain. The goal of the treatment study presented here was to further validate our model and emphasize the involvement of the HPA axis and BDNF signaling in the behavioral alterations observed after RUS. Future experiments should be planned to explore the impact of different pharmacological agents, dosages and routes of administration to ensure maximum drug delivery to the brain. In addition, exploring the outcomes at more chronic timepoints and after longer treatment durations is imperative to ensure better translational outcomes.

Chapter 6. Discussion and future directions

6. 1 Summary of research findings

PTSD is a debilitating condition affecting the lives of 13 to 24 million Americans and 2 to 4 million in the United Kingdom. The number of PTSD patients is expected to increase in both military personnel and civilians because of ongoing military interventions, refugee crises and the increasing number of terrorist attacks and mass violence (Richter-Levin et al. 2018). The past decade has witnessed a growing awareness and acknowledgment of the gravity of this condition and its detrimental consequences. However, no disease-modifying drugs are currently available due in part to our current lack of understanding of the pathophysiology of PTSD (Krystal et al. 2017). PTSD develops in a fraction of individuals after exposure to a significant stressful event (Kessler et al. 1995) and is accompanied by a number of changes in the HPA axis such as low baseline glucocorticoid levels and increased sensitivity to feedback regulatory mechanisms (Daskalakis, Lehrner, and Yehuda 2013; Yehuda 2001; Yehuda et al. 1995). Because the changes in the HPA axis are persistent, and correlate with PTSD symptom severity, stress homeostasis mechanisms are thus believed to be chronically impaired in PTSD (Fragkaki et al. 2016; Nijdam et al. 2015; Watkins et al. 2016). Several ethical and methodological challenges preclude the study of these mechanisms in humans, therefore preclinical animal research provides an alternative platform that is better suited to conduct such studies under controlled conditions. The challenge, however, is to develop an easily reproducible stress procedure capable of generating maladaptive responses that disrupts stress regulatory mechanisms in animals, which persists many months after the last stress exposure. In addition, for these procedures to be etiologically relevant to human PTSD and demonstrate

construct/face validity, they must also result in multiple chronic PTSD-like behavioral phenotypes. The first aim of this thesis was to address these challenges by examining the chronic effects of a combination of social, physical and psychological stress procedures in mice. Our stress paradigm combined multiple types of traumatic exposures including predator odor under restraint, footshocks, social instability and social isolation.

I have demonstrated in chapter 2 that our stress model generated a number of PTSD-associated behavioral changes that were correlated with decreased baseline plasma corticosterone and ACTH levels and diminished FKBP5 expression in the hypothalamus. These results implicate a blunted HPA axis function, which is a key neurobiological finding in human subjects with PTSD. This model also shows a chronic reduction in hippocampal CA1 volume and BDNF levels in addition to early, short-term changes in amygdaloid synaptic plasticity markers.

A number of interesting questions emerged from the findings in chapter 2, which were mainly recorded at 6 months after RUS. The first set of questions is centered around the HPA axis findings: 1) Why would animals exposed to our stress procedures show low baseline plasma corticosterone levels? Is there an attenuation in the pituitary response to CRH stimulation from the hypothalamus? Additionally, since FKBP5 levels were lower in the hypothalamus, is the GR negative feedback sensitivity altered in the hypothalamus and possibly other brain regions? The second set of questions concerns the time-dependent changes observed in several stress-related markers after RUS exposure.

To address the first set of questions, I conducted HPA axis challenges on stressed mice at one month after RUS, as described in chapter 4. The combined dexamethasone suppression and CRH stimulation test is often used to examine pituitary function and has

been shown to have better sensitivity in diagnosing pituitary-related disorders relative to the dexamethasone suppression test (Leistner and Menke 2018). Stressed animals showed normal plasma ACTH levels and lower plasma corticosterone levels in response to the DEX-CRH test. This indicated that the pituitary response is normal, and the attenuation in corticosterone may be due to decreased adrenal responsiveness. Stressed animals also showed lower corticosterone levels in response to the DEX-ACTH test, which examines adrenal function. These findings lead us to the conclusion that the observed low baseline corticosterone levels are likely due to mild adrenal insufficiency in stressed animals. My findings can also be explained by increased sensitivity of adrenal GR autoreceptors to dexamethasone suppression. I opted to use the combined DEX-ACTH test to ensure that endogenous corticosterone and ACTH levels are suppressed. However, additional experiments using only ACTH stimulation without DEX administration or prior testing would help us to better understand the adrenal function in stressed mice. Stressed animals showed decreased nuclear localization of GR receptors in the hypothalamus at baseline and after acute stress relative to control animals (Chapter 4), further implicating an impaired negative feedback mechanism and possibly prolonged chronic HPA axis activation in the hypothalamus. The source of this overactivation remains elusive but may be partly attributed to dysregulation in the input from the prefrontal cortex and limbic regions such as the amygdala and hippocampus involved in the allostatic management of stress. Interestingly, CRH-R1 levels were upregulated in stressed animals after acute stress challenge and circulating ACTH levels showed a trend towards an increase, which is consistent with central HPA axis hyperfunction. Taken together, our data suggest that there is decreased glucocorticoid negative feedback at the level of the hypothalamus resulting in prolonged HPA axis activation. However, due to the attenuated adrenal response to ACTH

stimulation, this HPA axis hyperactivation does not seemingly result in an increase in circulating plasma corticosterone levels, possibly owing to impaired ACTH mobilization of corticosterone from its precursors or inadequate GR autoreceptor feedback mechanism in the adrenal gland. This unique stress response is an important finding to advance our understanding of stress disorders in general, and it could also be relevant to the subpopulation of PTSD patients who demonstrate low baseline plasma corticosterone levels but a normal response in the dexamethasone suppression test.

The second topic that emerged from chapter 2 was the time-dependent changes observed in several markers. For instance, synaptic plasticity markers such as CAMK, PSD95, and GluN1 were altered in the amygdala at only the acute timepoint (10 days after RUS), while levels of other markers, such as hypothalamic FKBP5 and hippocampal CRH, were altered only at 6 months. These observations are probably driven by the differential effects of RUS and social isolation on these markers. The changes in amygdala synaptic markers could be transient and exclusively dependent on some of the stress procedures applied during RUS, such as restraint stress, TMT exposure, and footshocks. Therefore, a number of these markers were dysregulated at the acute timepoint but did not persist for any chronic period after RUS cessation. On the contrary, it is possible that social isolation (SI) is essential for the downregulation of FKBP5 in the hypothalamus and upregulation of hippocampal CRH, as these alterations were not observed at the acute timepoint (i.e. when mice had only experienced 10 days of SI).

Since one of the primary goals of developing this mouse model is utilization in drug discovery studies, I performed our treatment studies at one month after RUS exposure to minimize the lag time in future drug screening experiments. Examining the stress effect at the one-month timepoint is a valid representation of the chronic nature of stress given

that the DSM-5 criteria mandate the persistence of PTSD symptoms for at least one month in humans. In addition, because the average lifespan of mice is 30-40 times shorter than that of humans, persistence of behavioral deficits in mice for more than one month represents a longer relative duration in their life. In chapter 5 I performed a pilot treatment study aiming to restore hippocampal BDNF levels and recover normal HPA axis function which were chronically affected in our model. Animals were either given DEX, DHF (TrkB receptor agonist) or a combination of both to examine the impact of these treatments on stress-related behaviors. The choice of daily continuous exogenous supply of compounds in drinking water, particularly for DEX, was designed to correct alterations in tonic and pulsatile actions of corticosterone in our model, as diurnal fluctuations in the circadian rhythm of corticosterone is known to affect its allostatic regulation of stress. Chronic DEX treatment attenuated the recall of contextual fear memories, while DHF improved their anxiety-like behavior. The combination group emerged as the best modality in restoring homeostasis. This study exemplifies the utility of our model in future PTSD treatment studies.

Mild traumatic brain injury is a worldwide public health problem, with at least 41 million people affected per year (Gardner and Yaffe 2015). Mild traumatic brain injury is known to increase the risk for psychiatric disorders and neurodegenerative diseases, especially with repeated exposures. Active duty soldiers often suffer from comorbid mTBI and PTSD, which can be clinically challenging to diagnose due primarily to the heterogeneity and clinical overlap shared by both conditions. In addition, the impact of mTBI on PTSD symptom severity is not clearly understood. This emphasizes the critical need to develop a relevant animal model to examine the chronic consequences of comorbid PTSD and TBI. In chapter 3, I examined the impact of repetitive mild TBI (r-mTBI) on the

behavioral outcomes in our stress paradigm. Repetitive mild traumatic brain injury procedures (5 injuries with a 48-hour inter-injury interval) were administered concurrently with the 21-day RUS procedures to reproduce the nature of trauma endured by soldiers during a single deployment. The impact of repeated deployment on the severity of PTSD and mTBI, either individually or comorbidly, has received little attention in either clinical or preclinical studies. Therefore, I also examined the effects of repeat exposure to the procedures of RUS and r-mTBI on the chronic behavioral and neurobiological outcomes in mice.

I observed that after repeated exposure to RUS, stressed animals showed intensified contextual and cued fear memory recall and deficits in long-term recall of spatial memory. Exposure to the 5-injury r-mTBI model in combination with RUS, either once or twice, was associated with very similar outcomes, including: spatial memory impairments and reduced immobility time in the cued fear memory and forced swim tests relative to stressed animals. The changes in cued fear memory stress-coping behavior after r-mTBI may be driven by the hyperlocomotor activity observed after r-mTBI and may also involve an underlying complex behavior arising from the interaction between two opposite behavioral domains. Furthermore, I reported complex biological outcomes after repeated RUS and r-mTBI that involved reduced spine density and dysregulation in NMDA receptor levels 3 months after exposure to stress or r-mTBI. A summary of the findings presented in this thesis is presented in **Tables 6.1** and **6.2**.

	Fear memory recall	Anxiety-like behavior	Stress-coping strategies	Spatial memory
One RUS Exposure	Contextual (one month after RUS, abrogated by DEX treatment) Cued (6 months after RUS)	One month after RUS (abrogated by DHF treatment)	Passive stress-coping at 6 months after RUS	No change
One RUS+r-mTBI Exposure	Contextual (No recall at any timepoint) Cued(only at the acute timepoint)	No change relative to control animals	No change relative to control animals	r-mTBI-related deficits at 6 months after RUS
Two RUS Exposures	Contextual (3 months after the second RUS) Cued((3 months after the second RUS)	Acute timepoint	Passive stress-coping at 3 months after the second RUS	Deficits in long-term spatial memory recall at 3 months after initial training
Two RUS+r-mTBI Exposures	Contextual (3 months after the second RUS) Cued (Acute timepoint)	No change relative to control animals	No change relative to control animals	r-mTBI-related deficits at 3-months after the second RUS

Table 6.1. A summary of the main behavioral experiments presented in this thesis.

	HPA axis function	Hippocampus structure	BDNF	Synaptic plasticity
One RUS Exposure	Low baseline plasma corticosterone levels (14, 30, and 180 days after RUS) Low ACTH levels at 6 months after RUS Low plasma corticosterone levels in response to acute stress, DEX/CRH and DEX/ACTH	Decreased hippocampal CA1 volume at 6 months after RUS	Decreased hippocampal BDNF levels at 6 months after RUS	No change in hippocampal or amygdalar markers at 6 months after RUS Acute changes in the amygdalar markers (10 days after RUS)
Two RUS Exposures	No change in corticosterone or ACTH levels at the acute timepoint and at 3 months after RUS	Reduced spine density in the hippocampus (CA1) at 3 months after the second RUS	No change in hippocampal BDNF levels	Reduced PSD95, CAMKII, GLUN2A/GIUN 2B ratio at 3 months after the second RUS (hippocampus)
Two RUS+r-mTBI Exposures	No change in corticosterone or ACTH levels at the acute timepoint and at 3 months after RUS	Reduced spine density in the hippocampus (CA1) at 3 months after the second RUS	No change in hippocampal BDNF levels	Reduced CAMKII, GLUN2A/GIUN2B ratio at 3 months after the second RUS (hippocampus)

Table 6.2. A summary of the main neurobiological findings in this thesis.

6. 2 Study limitations and future directions

The work presented in this thesis to has significantly extended the existing literature, however I acknowledge that there are a few limitations in the current model and therefore will be proposing additional experiments to address these in the future.

Addressing individual variability

Not all individuals exposed to a traumatic event develop PTSD, which can be explained by individual variability in stress resiliency attributed to genetic and epigenetic factors, particularly prior risk factors, such as childhood abuse or lack of social support. To account for the variability in stress response in our model, additional experiments using larger sample sizes should be planned. Subsequently, animals exposed to the RUS paradigm should be subdivided into susceptible and resilient groups based on their cumulative scores in multiple behavioral experiments. This approach is very similar to the procedures used to diagnose PTSD in humans, such as PCL-5 and CAPS, in which an individual is assigned a score based on the cumulative symptom severity. Biomarker discovery and genetic experiments can then be conducted on subgroups to explore the differences between the two populations. As depicted in **Figures 2.4E** and **2.4F**, there are 3 or 4 animals that show significantly higher freezing scores within the RUS group at 3-month and 6-month timepoints, which suggests that our model can potentially be utilized to explore individual variability in response to stress.

Another limitation to our studies is the lack of a clear understanding of the impact of each stressor on the observed phenotypes. In chapter 4 I showed that social isolation alone can produce HPA axis alterations that are very similar to the ones observed in our

RUS model, and therefore it is a key component in our stress procedures. In addition, the ability of stressed mice to recall fear memories 6 months after RUS (Chapter 2) also suggests that our fear conditioning procedures are critical to the paradigm. However, the impact of the 10 repeated exposures to TMT under restraint and unstable social housing during the 21-day RUS paradigm is not clear. Additional experiments comparing the outcomes of exposure to the RUS paradigm with and without any of these components should help elucidate their impact and possibly reduce the duration of the RUS paradigm. For example, if the deficits observed after our stress paradigm are comparable using 5 or 10 exposures to TMT under restraint, the paradigm duration can be shortened to 14 days instead of 21, which would decrease the delay between stress exposures and testing, facilitating more high throughput studies in the future.

Exploring the impact of sustaining an earlier mTBI on stress-related phenotypes

In chapter 3, I examined the impact of concurrent exposure to r-mTBI and RUS on the behavioral outcomes in both conditions. In the context of human PTSD and TBI, these experiments are valuable in understanding the complexity of symptoms presentation in the comorbid condition. However, our experiments did not examine whether prior mTBI increases the risk to PTSD. This issue is highly relevant to the military, due to high number of mTBI sustained by soldiers during training prior to deployment. To address this issue in our model, future experiments should involve receiving the 5-hit-mTBI model one or three months before exposure to the RUS paradigm followed by behavioral and molecular analysis at extended timepoints. Although few preclinical studies have examined the effects of earlier TBI on fear conditioning and anxiety-like behavior in stress models (reviewed in Chapter 1), none of these studies were performed on a chronic timescale like the studies presented here.

Chronic hypopituitarism, or decreased pituitary function, has been reported in approximately 16% of mTBI patients (Tan et al. 2017). The exact mechanism by which hypopituitarism evolves after mTBI, and its effect on recovery times, is not clearly understood (Tanriverdi and Kelestimur 2015). Because a single exposure to the RUS model resulted in a significant change in HPA axis function, future studies should also examine the pituitary function after r-mTBI alone and after combined exposure to r-mTBI and RUS. These studies should be done after an acute stressor or HPA axis challenge to clearly elucidate the r-mTBI-related alterations. Because behavioral testing of laboratory animals is known to activate the HPA axis and increase the levels of plasma corticosterone (Arndt et al. 2009), attenuation in pituitary function and subsequent decrease in plasma corticosterone levels may affect the behavior of r-mTBI animals. These studies may also shed the light on the disinhibition behavior observed after r-mTBI.

Understanding the role of FKBP5 in regulating GR activation.

FK506 binding protein (FKBP5) has received a lot of attention after the discovery of its contribution to GR resistance, observed in squirrel monkeys (Denny et al. 2000; Reynolds et al. 1999). Although squirrel monkeys show 50 times higher free cortisol levels than humans, no signs of hypercortisolism are observed in these animals due to FKBP5 overexpression (Binder 2009; Hartmann et al. 2012; Merkulov et al. 2017). Multiple reports have shown that changes in FKBP5 polymorphisms leading to dysregulation in its functionality are involved in several stress and anxiety disorders, including PTSD (Binder 2009). The role of cytoplasmic FKBP5 in the translocation of GR receptors and its implications in GR resistance have been extensively studied. However, several questions regarding the function of nuclear FKBP5 and the **role** of the single nucleotide polymorphisms associated with FKBP5 in mood disorders remain unanswered. In chapter

4, I showed unique alterations in FKBP5 levels in stressed and socially isolated mice that can potentially be used to understand FKBP5 functions. These experiments were limited because I have only examined a single timepoint after acute stress (forced swim). A clearer understanding of regulation of the GR ultra-short feedback loop involving FKBP5 can be achieved by sampling mRNA and protein expression levels of different components of that loop (GR, FKBP51, FKBP52, HSP90) at different timepoints after acute stress or with variable intensity of the acute stressor (longer forced swim durations). The rate of expression can then be extrapolated and compared in control and stressed animals to highlight the differences. In addition, experiments exploring SNPs in the FKBP5 gene and posttranslational modifications and changes in FKBP5 expression levels in susceptible and resilient mice can also be conducted either at baseline or after acute stress. A comprehensive analysis of these data can help elucidate the functions of FKBP5, especially in the context of suitability and resilience to PTSD. This work can also be corroborated by conducting cell culture studies in iPSC derived hypothalamic cell lines from PTSD patients harboring related genetic polymorphisms, exposed to different elevated corticosterone concentrations to mimic an acute stress response.

In conclusion, the work presented in this thesis provides a laboratory PTSD model with face and construct validity, paving the way for future studies to better understand the pathophysiology of PTSD and providing a relevant platform for interrogation of novel, targeted, therapeutic approaches which are urgently needed to combat the current and growing PTSD patient population.

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